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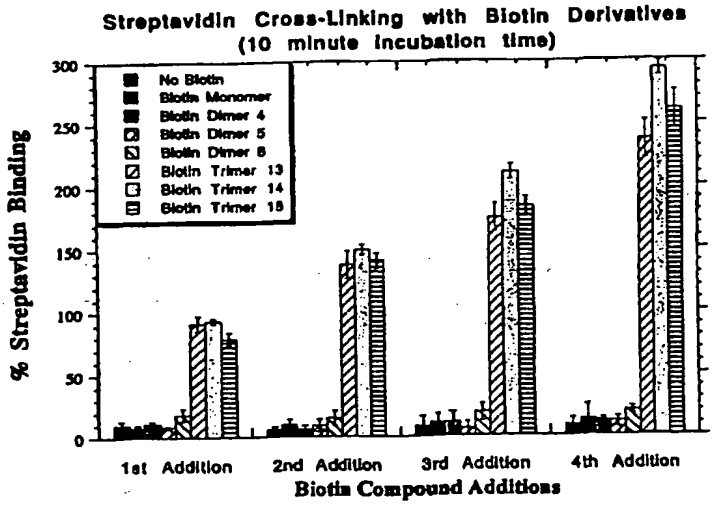

(57) Abstract

Water soluble biotin-containing compounds and biotinylation reagents incorporating soluble linker moieties are disclosed. The water soluble biotin-containing compounds and biotinylation reagents may additionally comprise one or more moieties that confers resistance to cleavage by biotinidase or that is cleavable in vitro or in vivo. Biotin-containing compounds and biotinylation reagents may include a reactive moiety that provides a site for reaction with yet another moiety, such as targeting, diagnostic or therapeutic functional moiety. Conjugates including functional moieties are disclosed. Biotin dimers, trimers and multimers comprising water soluble linker moieties which demonstrate enhanced water solubility are also disclosed. Methods for amplifying the number of sites for binding biotin-binding proteins at a selected target using biotin trimer compounds are disclosed. Methods for synthesizing the biotin-containing compounds and biotinylation reagents, and methods for using such compounds are described and an example of the invention, streptavidin cross-linking with biotin derivatives is shown in the figure.

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BIOTIN-CONTAINING COMPOUNDS, BIOTINYLATION REAGENTS AND METHODS

Reference to Priority Application

This application claims priority from U.S. Provisional Patent Application No. 60/011,321, filed February 8, 1996, entitled Water Soluble Biotin Derivatives.

Field of the Invention

The present invention relates generally to biotin-containing compounds, biotinylation reagents, and methods for synthesizing such compounds and reagents. The present invention relates, more specifically, to biotin-containing compounds and biotinylation reagents suitable for *in vitro* and *in vivo* applications that are soluble in aqueous solution. The biotin-containing compounds and biotinylatin reagents may additionally comprise constituents that confer other properties or functionalities, such as biotinidase-stabilizing properties, cleavable moieties, reactive moieties, and functional moieties, including diagnostic or therapeutic moieties such as dyes, radionuclides and drugs, targeting moieties, and the like. Finally, the present invention also relates to methods for synthesizing and using such biotin-containing compounds and biotinylation reagents.

Background of the Invention

The very strong interaction of biotin, illustrated below as chemical structure **1**, with the proteins avidin and streptavidin renders biotin compounds useful for numerous applications. For example, many diagnostic tests use biotinylated derivatives. The widely used enzyme-linked immunosorbent assay (ELISA), which was developed as an alternative to radioimmunoassays, employs biotinylated antibodies. Other biotinylated

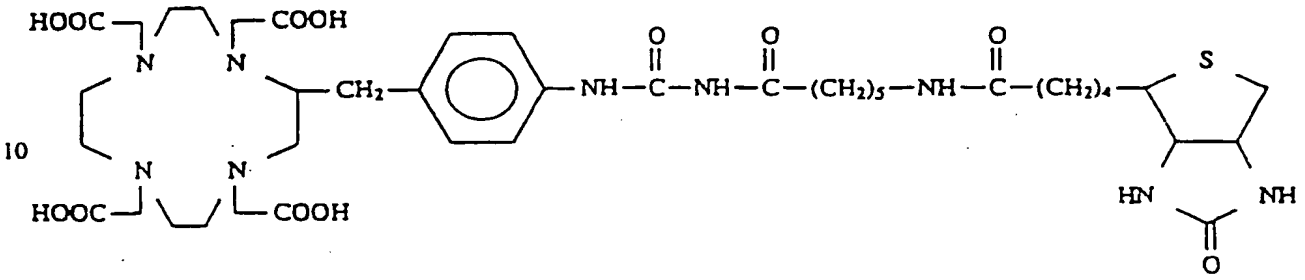
compounds have been used as probes. Biotinylated nucleic acids have also been widely used. Purification techniques such as affinity chromatography frequently employ biotinylated materials.

More recently, biotin derivatives have been used in diagnosis and therapy of human disease. Notably, investigators have shown that use of a combination of monoclonal antibodies, streptavidin and/or avidin, and radiolabeled biotin derivatives improves the diagnostic and therapeutic characteristics of the radiolabeled monoclonal antibody tumor targeting system. One important application under investigation by a number of research groups is the use of "pretargeted" monoclonal antibody conjugates using the biotin/(strept)avidin ligand/anti-ligand pair for imaging and therapy of cancer.

One disadvantage of using biotin derivatives for many of these applications is their generally low solubility in aqueous media. Biotin derivatives and biotinylation reagents generally need to be solubilized in organic solvents, or media comprising a substantial level of organic constituents. Insolubility of biotin derivatives and biotinylation reagents in aqueous solutions is particularly problematic for *in vivo* applications where organic solvents cannot be used.

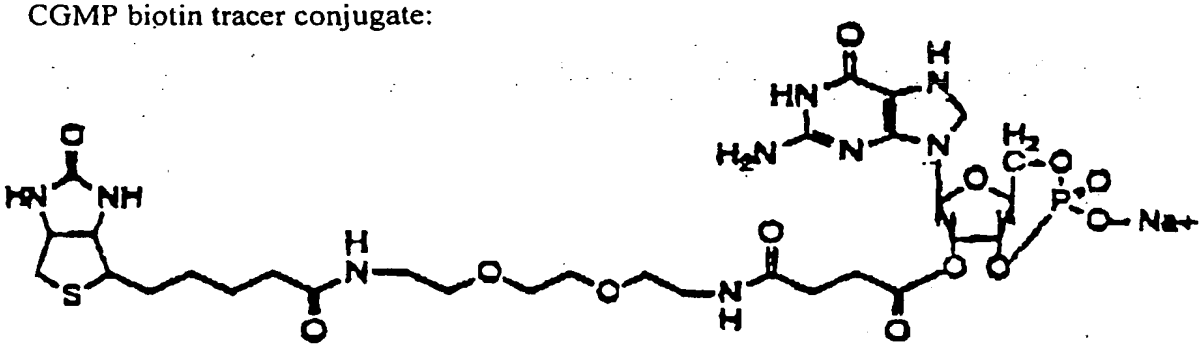
It has been recognized that the availability of biotin derivatives for binding with avidin- and streptavidin-containing compounds may be greater when a spacer molecule is used between the biotin moiety and the other moieties to which it is attached. The spacer molecules used have generally been molecules having a low solubility in aqueous media, such as aliphatic molecules. Such linkers generally reduce the aqueous solubility of the biotin-containing compound. In addition to causing solubility problems, the lipophilic nature of the biotin derivatives with linkers of low water solubility may cause them to associate with blood components, rendering their biological half-life in *in vivo* applications longer than desired. When biotin derivative-linker adducts are conjugated to another water soluble moiety, the soluble moiety generally confers greater water solubility to the conjugate as a whole. This approach provides a biotin-containing compound having enhanced water solubility, but the low solubility and hydrophobic nature of the linker reduces the availability of biotin for binding to avidin or strepavidin-containing compounds.

U.S. Patents 5,541,287 and 5,578,287 disclose compositions for use in pre-targeted delivery of diagnostic and therapeutic agents that employ biotin/avidin as the ligand/anti-ligand binding pair. These patents disclose that the 1,4,7,10-tetraazacyclododecane-N,N',N",N'"-tetra acetic acid (DOTA)-biotin shown below has been reported to have desirable *in vivo* biodistribution and is cleared primarily by renal excretion.



These patents also report that serum stability of the above conjugate, comprising a "long chain" linker including an aminocaproyl spacer, was observed to be problematic, while a "short chain" linker exhibited significantly improved serum stability. Numerous linkers for DOTA-biotin conjugates are disclosed in these patents. One of the patents discloses that sulfonates, which are fully ionized at physiological pH, improve water solubility of chelate-biotin conjugates. These patents furthermore disclose that drug-biotin conjugates that structurally resemble biotinyl peptides are susceptible to cleavage by biotinidase. Poor in vivo stability therefore limits the use of such conjugates in therapeutic applications. General strategies for improving serum stability are disclosed.

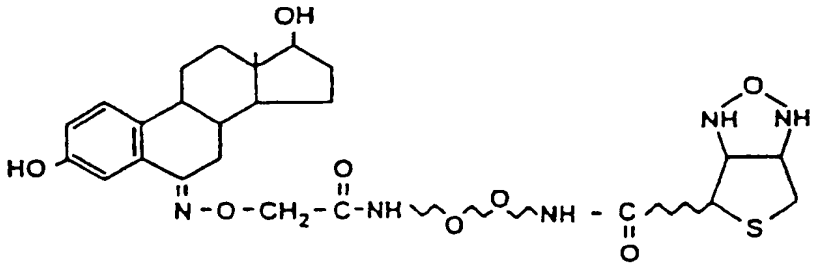
Dressendorfer et al. propose the following chemical structure for a succinyl-CGMP biotin tracer conjugate:



sc-cGMP-biotin

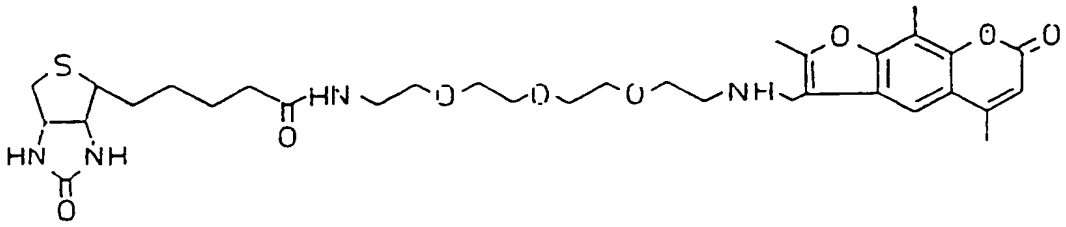
Dressendorfer et al., "A Non-Isotopic Immunoassay for Guanosine 3':5'-Cyclic Monophosphate Using a Cyclic GMP - Biotin Conjugate as a Tracer," Journal of Immunoassay 16(1), 37-53 (1995).

Mares et al. describe the synthesis and purification of the following biotin-estradiol conjugate:



Mares et al., "Synthesis of a Novel Biotin-estradiol Conjugate and Its Use for the Development of a Direct, Broad Range Enzyme Immunoassay for Plasma Estradiol," Journal of Immunological Methods 183 (1995) 211-219.

Levenson et al. disclose a synthesis for the biotinylated psoralen labeling reagent shown below:



The linker is referred to as a long, flexible hydrophilic spacer arm. Levenson et al., "Biotinylated Psoralen Derivative for Labeling Nucleic Acid Hybridization Probes," Methods in Enzymology, 184:577-587 (1990).

U.S. Patent 9,550,249 discloses water soluble biotin salts for oral, parenteral or topical administration. Salts of biotin with an aminoalcohol are disclosed, and biotinate of ethanolammonium is preferred.

U.S. Patent 4,478,915 discloses a process for applying layers of a protein and a ligand extender to a surface and to a multiple layer system. Biotin derivatives used as extenders include caproylamidobiotin and biocytin. Extenders such as fibrogen, albumin,

succinylated polylysine and ribonuclease modified with biotin or biotin derivatives are also disclosed.

Little attention has been devoted to the water solubility of biotin-containing compounds, or the synthesis of biotinylation reagents having improved solubility in aqueous solutions. Because biotin-containing compounds are increasingly being used in biological systems for diagnostic and therapeutic applications, where organic solvents cannot be used, there is a need for biotin-containing compounds exhibiting enhanced water solubility and improved linker systems. There is also a need for biotin-containing compounds that are resistant to cleavage by the serum enzyme biotinidase for use in *in vivo* applications.

Summary of Invention

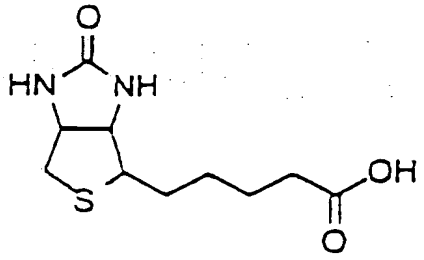
Biotin compounds (including modified biotin molecules) are conjugated with water soluble linker moieties to form biotin-linker adducts that are water soluble. Such biotin-linker adducts may additionally comprise one or more functional group that confers resistance to cleavage by biotinidase. The biotin-linker adducts or conjugated biotin compounds may also comprise one or more constituents that are cleavable *in vitro* or *in vivo*. Biotinylation reagents of the present invention comprise water soluble biotin-linker adducts linked to a reactive moiety that provides a site for reaction with yet another moiety, such as a targeting, diagnostic or therapeutic functional moiety. Conjugates including functional moieties are disclosed. Biotin dimers, trimers and multimers comprising water soluble linker moieties are also disclosed and demonstrate enhanced water solubility. Methods for synthesizing the biotin-containing compounds and biotinylation reagents, and methods for using such compounds are described.

Brief Description of the Drawings

The invention will be described below in with reference to the following detailed description and Examples, and the figures in which Fig. 1 illustrates the results of the experiment described in Example 14 demonstrating the successful cross-linking of streptavidin with biotin trimers.

Detailed Description

Naturally occurring biotin, illustrated below as structure **1**, is useful for many different applications as a result of its strong binding affinity for avidin or streptavidin. Many modified forms of biotin have been synthesized for various applications. Modifications of biotin at positions other than the carboxylate group, for example, provide molecules that have weaker interactions with avidin or streptavidin. Such modified biotin molecules, such as desthiobiotin, shown below as structure **2**, and biotin sulfone, shown below as structure **3**, are useful for some applications because they bind tightly enough to provide a strong association, yet they bind in a reversible fashion such that they can be displaced by tighter binding biotin derivatives. These modified biotin molecules (**2 and 3**) have low water solubility. Other modifications of biotin, such as conversion of the ureido functionality to an guanidinium functionality (e.g. iminobiotin, shown below as structure **4**) or conversion of the amide-NH by alkylation (e.g. methylation) or by acylation (e.g. acetyl) produce biotins of varying binding strengths.



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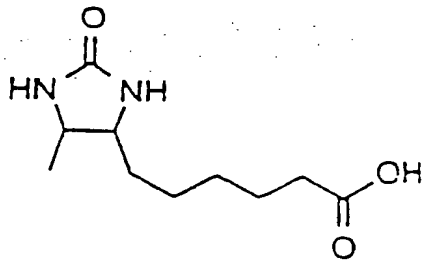

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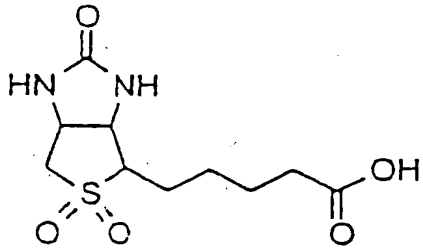

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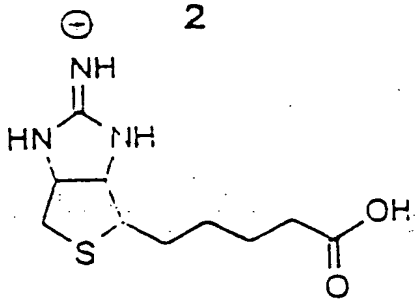


4

Modifications of biotin or biotin derivatives by reaction with amines of sterically bulky groups such as proteins (e.g. insulin) or steric small molecules such as branched chain moieties including 2-dimethyl-amino compounds or amino-aryl moieties are also

described above, and any other modifications to biotin or constituents thereof that bind to avidin or streptavidin or modifications to or constituents thereof.

The solubility of naturally-occurring biotin in water is approximately 0.2 mg/mL at neutral pH and ambient temperature. Modifications to biotin such as those described above generally reduce its water solubility, and biotin compounds having more than one biotin moiety per molecule are generally substantially insoluble in aqueous media. A biotin compound comprising two biotin moieties joined by an aliphatic 1, 12 diaminododecane linker moiety, for example, has such low water solubility that routine HPLC analysis does not detect any dimers in aqueous solution. Similarly, trimeric biotin molecules comprising aliphatic linkers exhibit water solubilities below normal detection levels.

The solubility of biotin moieties in aqueous media is enhanced according to the present invention by attaching a water soluble linker moiety, preferably through the carboxylate functionality of the biotin moiety. Water soluble linker moieties, according to the present invention, compromise any linker moiety that, when coupled to a biotin moiety, increases the water solubility of the biotin moiety. For purposes of this invention and the appended claims, the term "water soluble" used in connection with a linker moiety, biotin-containing compound or a biotinylation reagent indicates that the compound or reagent has a water solubility greater than that of naturally-occurring biotin: that is, greater than 0.2 mg/mL at neutral pH and ambient temperatures. Biotin-containing compounds and biotinylatin reagents of the present invention preferably exhibit water solubility of at least about 1 mg/mL at neutral pH and ambient temperature, and most preferably exhibit a water solubility of at least about 5 mg/mL at neutral pH and ambient temperature. Solubility may be ascertained by dissolving the compound in water, stirring the solution, and allowing the solution to stand at room temperature for about 24 hours. The solution is then centrifuged and the resultant aqueous layer analyzed using high-pressure liquid chromatography ("HPLC"). The HPLC analysis is conducted with a radiant using acetonitrile or methanol and water as the solvent mixture using a reverse-phase column. With some biotin compounds, a carboxylic acid or amine modifier is used in the solvent mixture. Solubility of a compound may alternatively be

determined using any of the techniques described in the Handbook of Solubility Parameters and Other Cohesion Parameters by A.F.M. Benton, CRC Press, 1983.

Water-soluble linker moieties preferably comprise hydrophilic moieties (e.g., polar functional groups) including electronically neutral and charged (i.e., ionic) moieties. Suitable hydrophilic moieties include electronically neutral moieties containing polar functional groups (i.e., groups that contain atoms of differing electronegativities such as organic compounds containing nitrogen, oxygen, and sulfur) that increase their hydrophilicity. Typically, these neutral hydrophilic moieties contain functional groups that hydrogen bond with water. Such hydrogen bonding groups include ether (-O-), hydroxy (-OH), amino ($-NR_2$, -NHR, $-NH_2$), and to a lesser extent thioether (-S-), and thiol (-SH) groups.

Other polar functional groups that may serve as hydrophilic moieties include ethers and carbonyl-containing groups such as acids, esters, amides, ketones, and aldehydes. Moieties that comprise multiple polar functional groups are more hydrophilic than those moieties that comprise a single polar functional group. Suitable moieties comprising multiple polar groups include, for example, polyhydroxy, polyamino, polyether, polyphosphoric acid, polyalcohol and polyamine moieties. Polyhydroxy moieties include, for example, glycols, glycerols, and polysaccharides including glucose, fructose, galactose, idose, inositol, mannose, tagatose, and N-methylglucamine. Polyalcohol moieties include, for example, N-methylglucamine and glucose derivatives. Polyether moieties include, for example, polyethelyne glycol, ethoxy ethanol, and ethoxy ethoxy ethanol. Polyamine moieties include, for example, spermine or spermidine.

Suitable charged hydrophilic moieties become either formally negatively or positively charged in water. Suitable negatively charged moieties include acid anions resulting from the dissociation of acids in water. For example, carboxylic acids ($-CO_2H$) dissociate to form negatively charged carboxylate ions ($-CO_2^-$) at pH greater than about 5. Other stronger acids such as phosphoric ($-PO_3H_2$) and sulfonic ($-SO_3H$) acids ionize to form phosphonate ($-PO_3^{2-}$) and sulfonate ($-SO_3^-$) anions, respectively, at pH greater than about 2. Other more weakly acidic moieties, such as phenols and thiols, may also dissociate to form their corresponding anionic derivatives that are also water solubilizing.

Depending upon the pH of the aqueous solution, abasic moieties may become formally positively charged moieties in water. These moieties become highly water soluble through protonation in aqueous solution. For example, at pH about 5, amines ($-NR_2$, $-NHR$, $-NH_2$) become ammonium ions ($-NHR_2+$, $-NH_2R+$, $-NH_3+$), which are water soluble moieties. Quaternary ammonium moieties ($-NR_3+$) are water soluble at all pHs. Suitable charged solubilizing moieties also include polylysine groups.

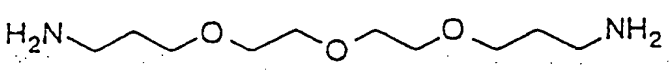
Water soluble linkers are preferably relatively linear molecules greater than 4 atoms in length, preferably between 6 and 50 atoms in length, and most preferably about 8 to 20 atoms in length. In one preferred embodiment, the linker is a linear molecule of 12-15 atoms in length. In the context of the present invention, the term "atom" refers to a chemical element such as C, N, O, S, or the like. The ranges provided herein are based on the relatively linear accounting of the linker. One of ordinary skill in the art will appreciate that a linker may be linear, branched, and may contain ring or cone structures.

Suitable water soluble linkers preferably comprise at least two coupling or reactive groups allowing the linker to bind to both a biotin moiety and another moiety, such as another biotin moiety, a reactive moiety, or a functional moiety. Suitable linkers may be homobifunctional, heterobifunctional, homotrifunctional, or heterotrifunctional. Homobifunctional agents may facilitate cross-linking, or dimerization of biotin moieties in a single step. Suitable water soluble homobifunctional, heterobifunctional, homotrifunctional, and heterotrifunctional linkers are available, and additional water soluble linkers may be prepared using commercially available linkers. Empirical factors such as the size (e.g., molecular weight and molecular conformation) and the nature (e.g., charge and constituency) of the biotin moiety or moieties, the reactive moiety or moieties, and the functional moiety it binds affect the choice of linker.

Homo- and heterotrifunctional linkers may be coupled to a functional moiety and a biotin moiety as described above, with the additional advantage of a third coupling site on the linker. One of ordinary skill in the art will appreciate that this allows for any number of different molecules to couple with the biotin moiety, including, for example, markers, such as radiolabeled and flourescent molecules; proteins and peptides, such as antibodies; and conjugating molecules. Suitable trifunctinal linkers include, but are not

limited to 5-N-Boc amino isophthoyl ditetrafluorophenyl ester; 3,5-diamino methyl benzoate; 5-(*p*-iodobenzoyl) amino-1,3-isophthaloyl ditetrafluorophenyl ester; 5-(*p*-tri-N-butylisoamylbenzoyl)-amino-1,3-isophthaloyl ditetrafluorophenyl ester; 4-(4-iodo)3-amidoethyl)-4'-2-(1,3-bistosyl)propyl phthalate.

Two principle types of water soluble linker moieties are preferred for use in the biotin-containing compounds and biotinylation reagents of the present invention. One type is a non-ionized linker which is made more soluble by functional groups such as ethers or hydroxyl groups. Non-ionized linker moieties render the biotin moiety more water soluble, while retaining the neutral character of the biotin moiety. Particularly advantageous non-ionized linker moieties comprise a diamino-ether moiety, such as the commercially available molecule 4,7,10-trioxa-1,13-tridecanediamine, shown below as structure 5, or 2,2'-(ethylenedioxy)diethylamine, shown below as structure 6, and tetraethylene glycol, shown below as structure 7. Diamino-thioether molecules are also advantageous in this application.



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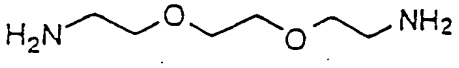

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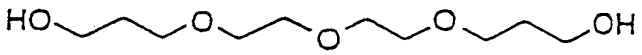

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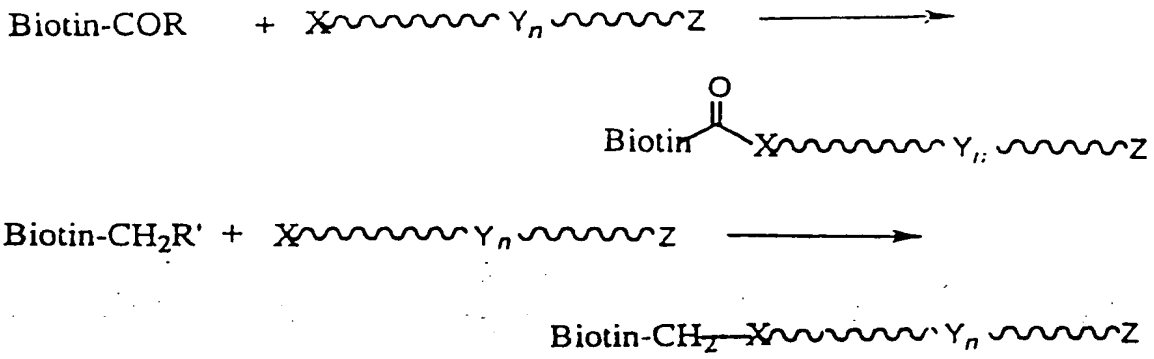
After activating the biotin moiety at a selected coupling site, water soluble linkers may be coupled to a biotin moiety to form a biotin moiety-linker adduct using any one of several means, including, for example, an amide forming reaction, employing an amine group on the linker and the carboxylate coupling site on the biotin moiety. Alternatively, a water soluble linker may be coupled to a biotin moiety through an amide forming reaction employing a carboxylate group on the linker and an amino group on a biotin moiety. The amide forming reaction may include the use of a coupling agent. Suitable coupling agents include carbodiimide coupling agents, such as 1-thyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 1-benzyl-3-(3-

dimethylaminopropyl) carbodiimide (BDC), 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide (CMC), and 1,3-dicyclohexylcarbodiimide (DCC).

Alternatively, the amide forming reaction coupling the linker to a water soluble biotin moiety may employ a reactive carboxylic acid group and an amine. Suitable reactive carboxylic acid groups include carboxylic acid derivatives that yield an amide upon reaction with an amine. Such reactive groups include, for example, any reactive carboxylic acid derivative, such as carboxylic acid halides, including acid chlorides and bromides; carboxylic acid anhydrides including mixed acetic anhydrides and triflouroacetic anhydrides; esters including, p-nitrophenyl esters, N-hydroxysuccinimide esters, and tetrafluorophenyl esters. Suitable techniques are described in detail in Bodanszky, Principles of Peptide Synthesis, Springer Verlag, Berlin, 1984.

Generalized formula for preparing non-ionized ether linking moieties, and two representative types of bonding (i.e. acyl or alkyl) to biotin, are illustrated below. The first reaction scheme depicts conjugation with the carboxylate (or activated carboxylate) of a biotin moiety. The second reaction scheme depicts conjugation wherein the biotin carboxylate functionality has been reduced and activated towards reactions with nucleophiles. The syntheses of representative biotin-containing compounds are set forth below in the examples.



where: R = good leaving group (e.g. tetrafluorophenol, p-nitrophenol, N-hydroxysuccinimide, etc.)

R' = good leaving group (e.g. Cl, Br, I, OTs, OMs, etc.)

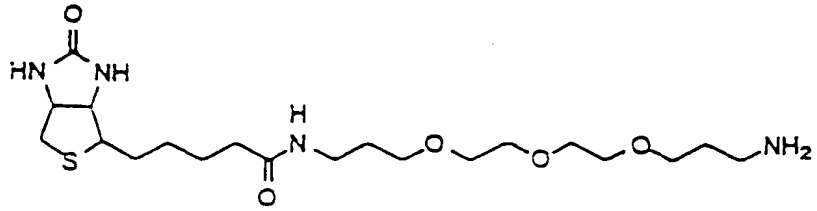
X = NH_2, OH, SH

Y = O, S

Z = NH_2, COOH, COR (where R is defined above), OH, SH, R' (as defined above)

n = 2 - 10

Some examples of biotin moiety-linker adducts comprising preferred water soluble linkers are shown below as structures 8 - 12.



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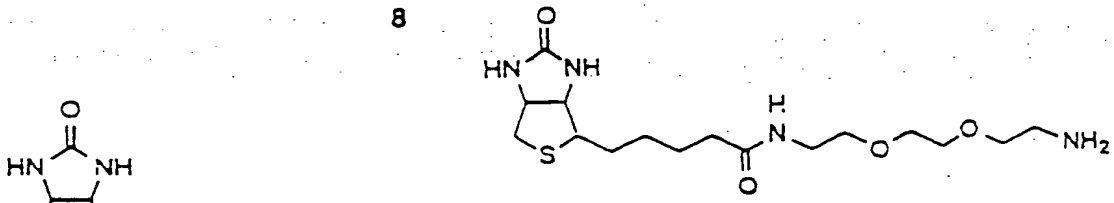

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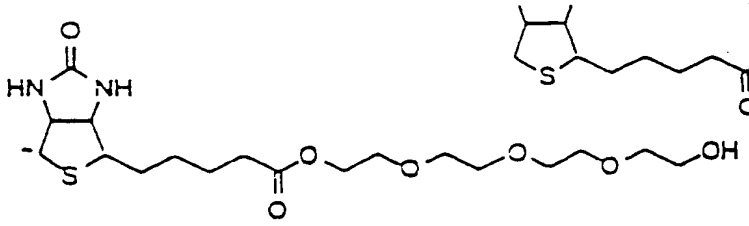

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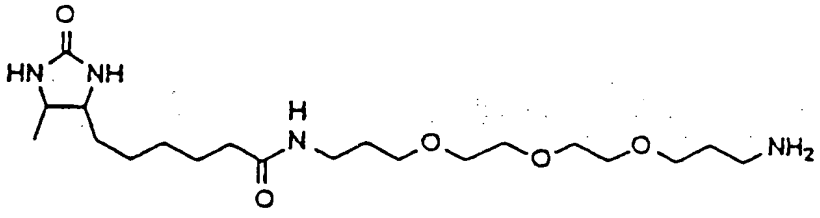

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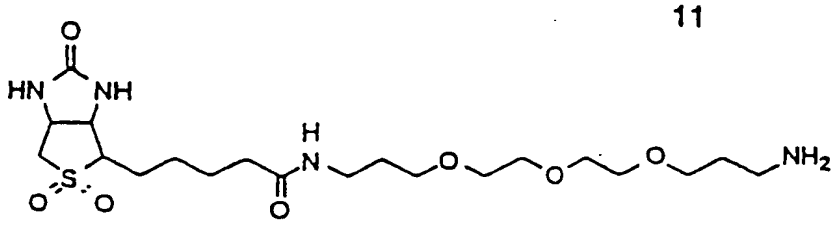

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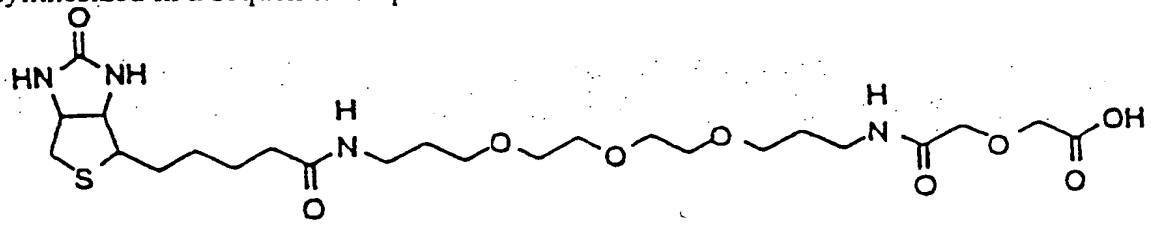
The water soluble linker moieties may also contain other functional groups attached to or within, the chain (e.g. amides) as illustrated in structures **13** and **14**, and may be synthesized in a sequential step of reactions.



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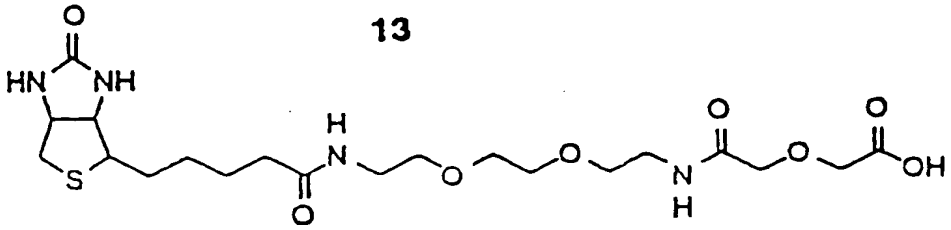
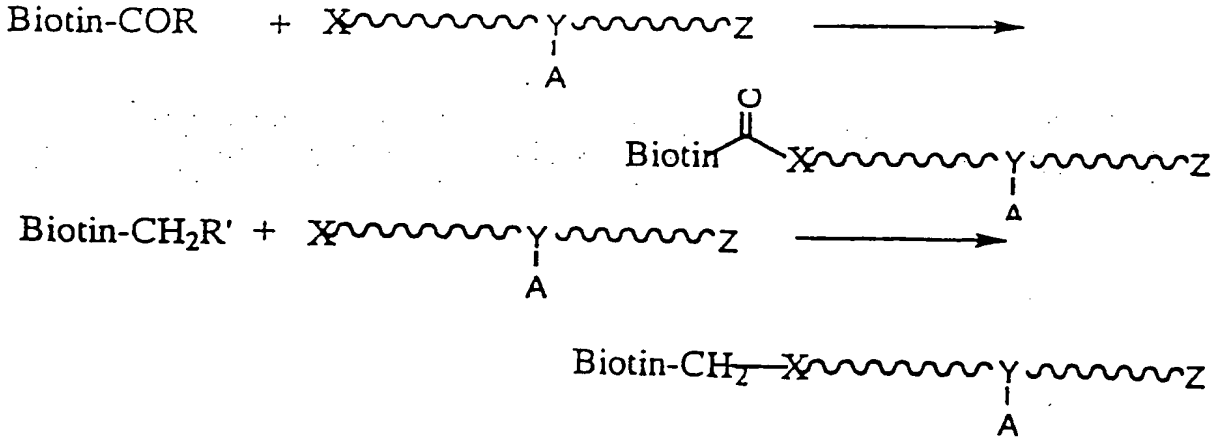


14

A second type of water soluble linker moiety comprises an ionized or ionizable moiety within the linker. The ionized functionality is preferably at least 3 atoms away from the point of conjugation with the biotin moiety. Functional groups containing a sulfonate or ammonium ion are advantageous. Linker moieties comprising anionic borane and carborane cage molecules are especially preferred, since these moieties provide biotin compounds having enhanced water solubility and a site for radiolabeling..

Representative schemes for preparing biotin moiety-linker adducts comprising ionic or ionizable functionalities are illustrated below:



where: R = good leaving group (e.g. tetrafluorophenol, p-nitrophenol, N-hydroxysuccinimide, etc.)

R' = good leaving group (e.g. Cl, Br, I, OTs, OMs, etc.)

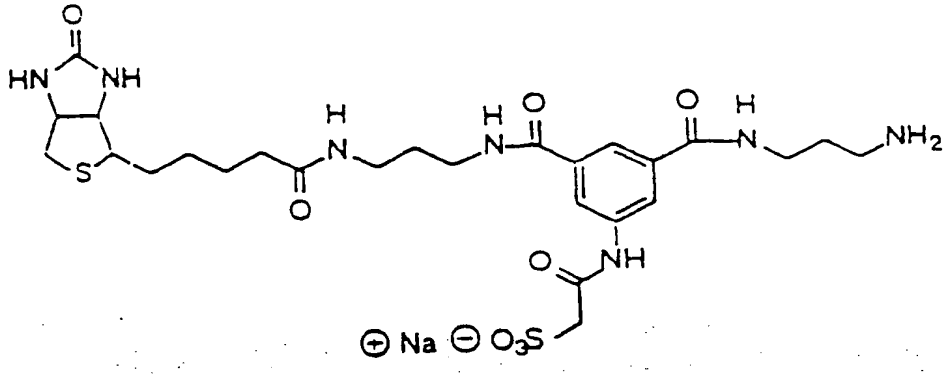
$X = NH_2$, OH, SH

Y = N, aryl,

$Z = NH_2$, COOH, COR (where R is defined above), OH, SH, R' (as defined above)

A = moiety containing an ionic or ionizable functional group

Structures 15 - 17 illustrate preferred biotin-linker adducts comprising an ionic or ionizable functional group. Other combinations of ionic or ionizable functionalities and linker moieties may be used (e.g. charged functionalities as part of aromatic ring substitution - aryl sulfonates, aryl ammonium ions) in combination with differing lengths of chains containing the ionic moiety branched from the linker moiety.



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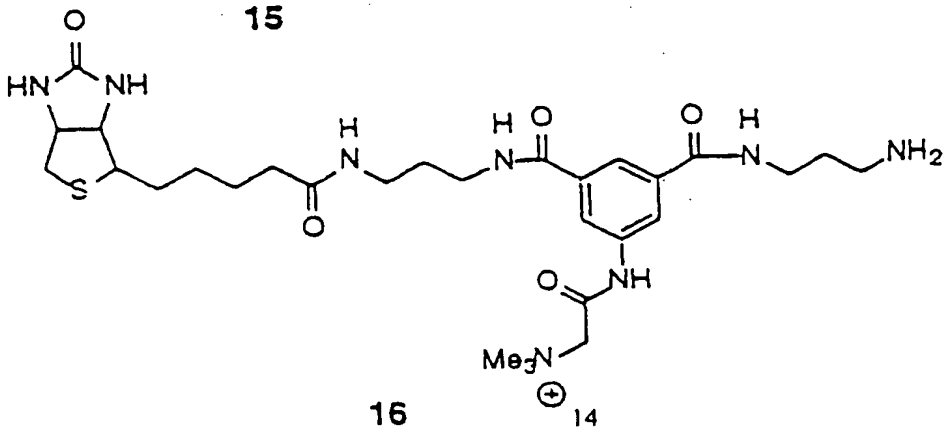

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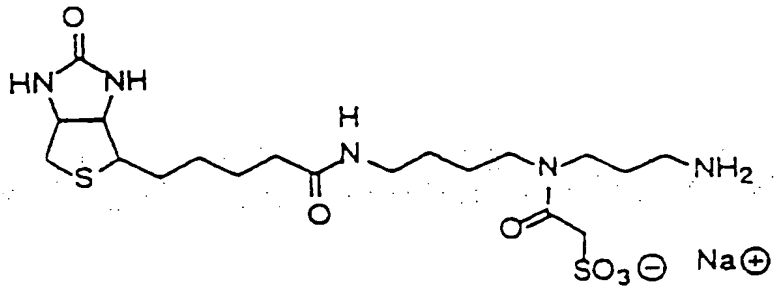
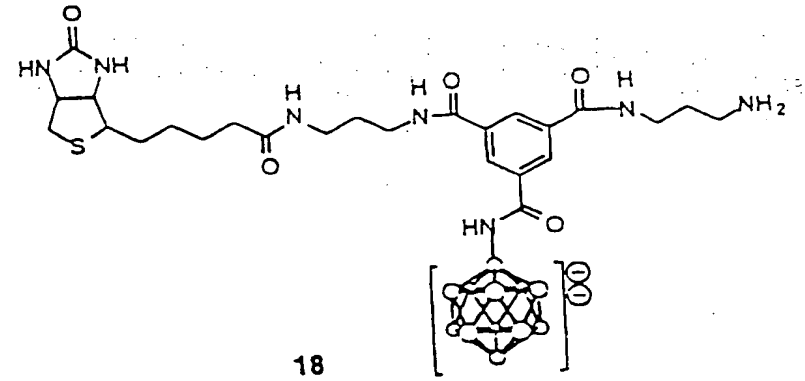


17

Another preferred ionic water soluble linker moiety contains anionic boron cage moieties. Structures **18** and **19,** shown below, comprise a dodecaborane (icosahedral) cage moiety which has a 2- charge, and structure **20** comprises a *nido*-dicarbon carborane cage moiety which has a 1- charge. Other anionic boranes or carboranes (*closo* or *nido*) may be used as water soluble linkers as well. The borane cage water solubilized linkers are preferably used when it is desirable to radiohalogenate the biotin compound.



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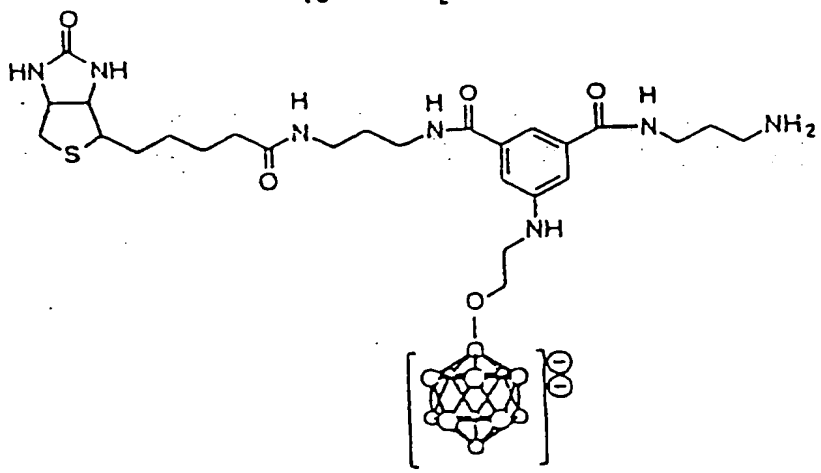

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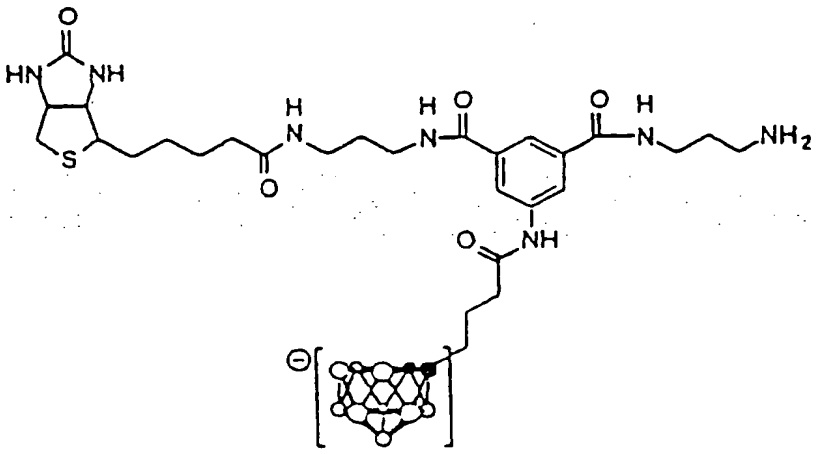
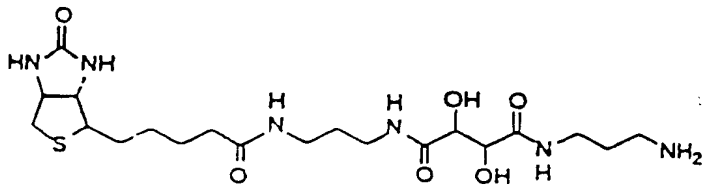


20

Yet another water soluble linker moiety comprises polyhydroxyl groups. Biotin-containing compounds illustrated below in structures **21** - **23** are exemplary of biotin-containing compounds comprising water soluble polyhydroxyl linking moieties. Linker moieties comprising from about 2-20 hydroxyl moieties may also be used, wherein the hydroxyl groups are bonded to the linking chain itself (as shown below in structures **21** and **22**), or are bonded to a branch point of the linker (as exemplified in structure **23**).



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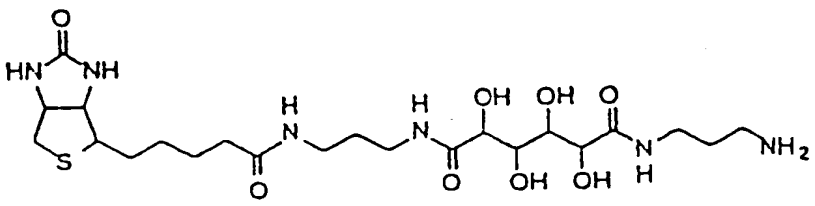

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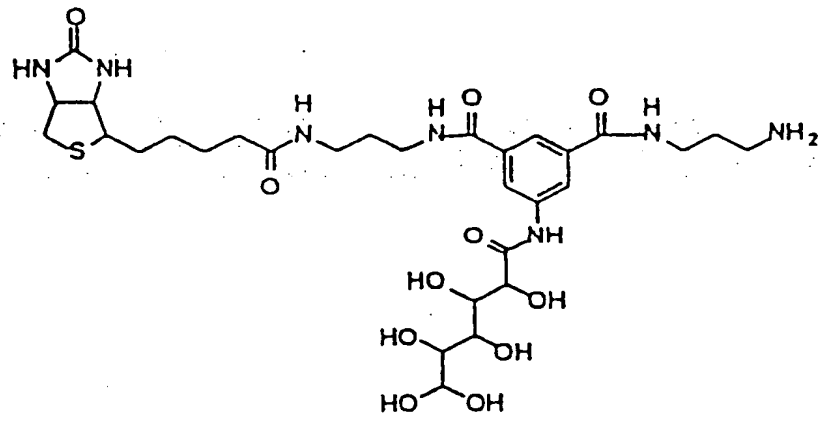


23

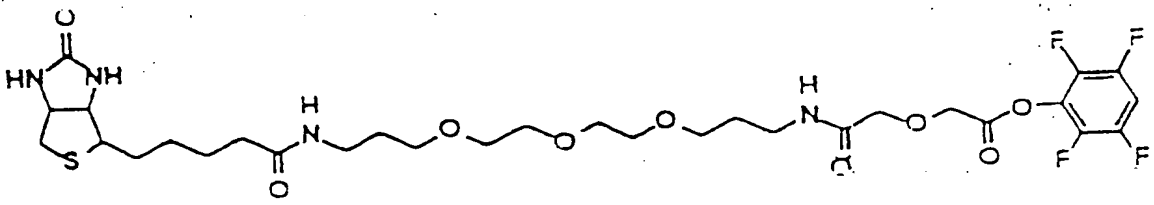
In some applications a cleavable linker is desired between the biotin moiety and the functional molecule, or, preferably, between the water soluble linker moiety bound to the biotin moiety and a reactive or functional group. In some situations, the cleavable functional group is prepared by the combination of a reactive moiety on the biotin compound with a functional group on a drug or reporter moiety. Compounds 37 and 38, illustrated below, are exemplary of this type of compound. Suitable cleavable linkers include disulfides, esters, imines and specific peptide sequences. Diol-ether containing linker moieties, such as triethylene glycol or tetraetheylene glycol, are used as cleavable linkers for many applications. These linker moieties can be used to will form an ester with the biotin carboxylate, rendering it cleavable, either *in vitro* or *in vivo*. Linker moieties comprising ether or hydroxyl functionalities, which also contain cleavable disulfide functionalities, are also preferred for some applications. For applications of biotin-drug conjugates, it is preferred that the cleavable linker release the drug in its bioactive form.

Modification of the linker moiety attached to the biotin moiety is desirable under certain circumstances to prevent the serum enzyme biotinidase from cleaving the water soluble linker from biotin. Introduction of a steric group alpha to the amine (or another functionality) of the linker which is attached to the biotin carboxylate provides resistance to cleavage by biotindase. Suitable steric moieties include carboxylates, larger alkyl

groups, aryl groups, heteroaryl groups and other groups in the same manner. Depending upon the steric bulk of the branching group alpha to the amine (or other) functionality attached to the carboxylate, some reduction in binding affinity for biotin-binding proteins may result. The particular application of the biotin compound determines how much steric bulk is desired or can be tolerated in the branched group. Modifications of biotin by conjugation with water soluble linkers possessing a branched chain alpha methyl (or other steric) group are desirable to produce conjugates that are more resistant to *in vivo* degradation by the enzyme biotinidase. Preferred alpha methyl group containing linkers include 3-aminobutyric acid, 1,2-diaminopropane, and 1,5-diaminohexane (Dytek A). By combining a variety of biotin moieties with carboxylate coupled steric moieties and a water soluble linker moiety, water soluble biotin compounds having varying binding affinities with biotin-binding proteins and enhanced resistance to *in vivo* degradation are provided.

It is also desirable to biotinylate various compounds such as small molecules, peptides, proteins, oligonucleotides, targeting moieties, diagnostic or therapeutic moieties, and the like. The biotinylation of these compounds is preferably accomplished by conjugation with an activated biotinylation reagent of the present invention comprising a water soluble linker with a reactive functionality. Suitable reactive moieties include amino reactive moieties such as carboxylate active esters including hydroxysuccinimidyl, hydroxybenztriazole, N-hydroxypyrrolidone, phenyl, 2- and 4-nitrophenyl, 2-chloro-4-nitrophenyl, 2-nitro-4-sulfophenyl, cyanomethyl, 2-mecaptopyridyl, 4-fluorophenyl, 2, 4-dichlorophenyl, trichlorophenyl, tetrafluorophenyl, tetrafluorothiophenyl, pentafluorophenyl, and the like; imidate esters such as methyl imidate esters and methyl benzimidates; isocyanates or isothiocyanates; alpha-haloacetamides such as alpha-iodoacetamides and alpha-bromoacetamides; aldehydes such as alkylaldehydes or benzaldehyde, and the like. Suitable reactive moieties may alternatively or additionally comprise sulfhydryl reactive moieties such as maleimides and alpha-haloacetamides; oxidized carbohydrate reactive moieties such as amines, hydrazines, acyl hydrazines, and hydroxylamines; and pH-, photo- or heat-activated reactive moieties such as arylazides, diazonium salts, and diazirines.

Exemplary biotinylatin reagents of the present invention comprising an activated ester (tetrafluorophenyl), **24**; a maleimide group, **25**; an iodoacetamide group, **26**; an acyl hydrazine group, **27**; a hydroxyl amine group, **28**; and a nitrophenylazide moiety, **29** are illustrated below. These compounds can be conjugated through amines (e.g. **24**, **25**), sulfhydryl groups (e.g. **25**,**26**), oxidized carbohydrate, ketone or aldehyde functionalities (e.g. **27**, **28**), or through a photoreaction. The compounds illustrated below are exemplary of many other reactive functionalities that can be coupled with biotin-linker moieties for application to biotinylation of molecules. Additional reactive functionalities are described in Wilbur, *Bioconjugate Chem.* 3, 433-470, 1992 and Bodanszky, Principles of Peptide Synthesis, Chapter II, pp. 28-35, Springer-Verlag, New York, 1984.



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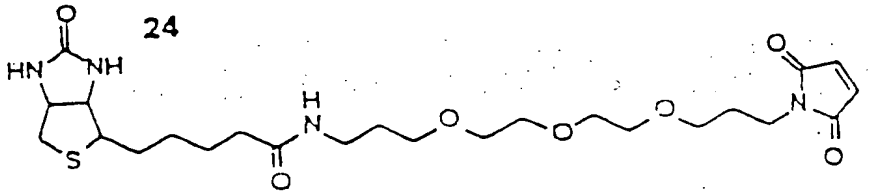

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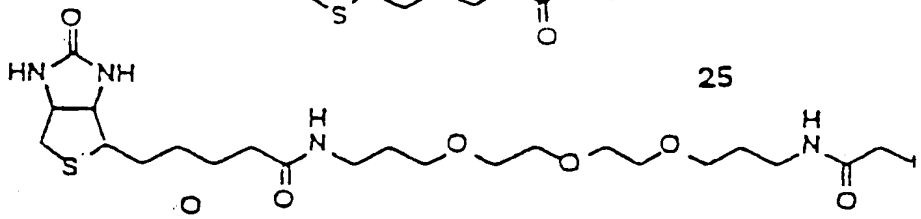

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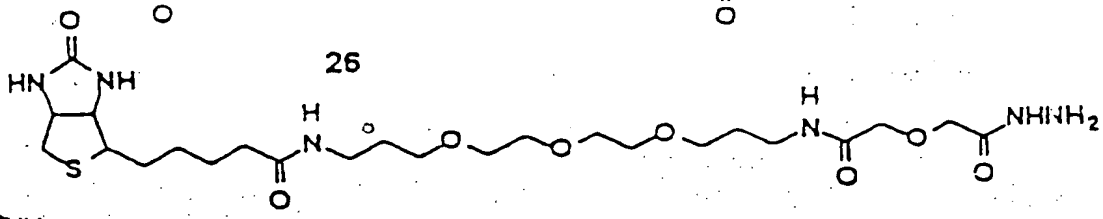

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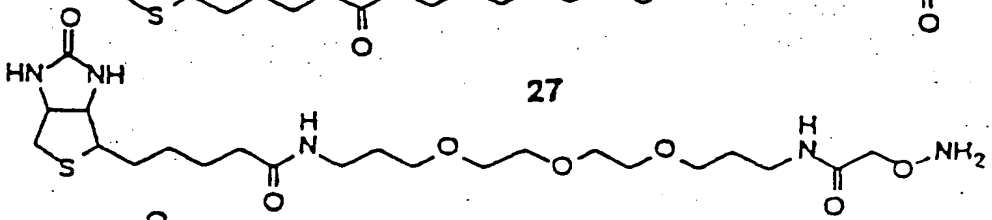

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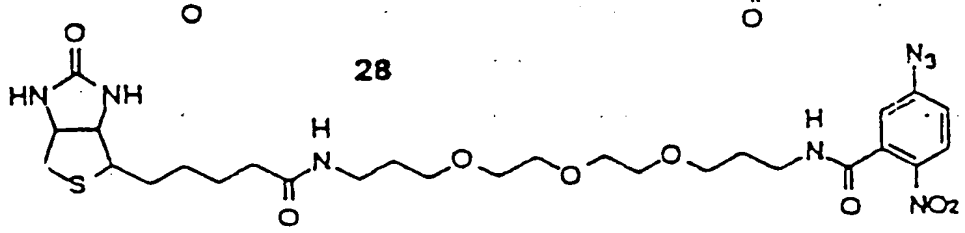


29

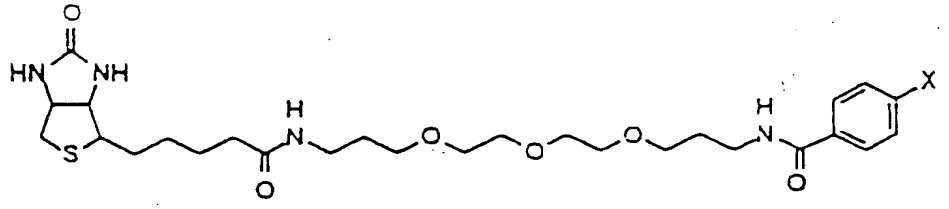
Many water soluble biotin-containing compounds may also be prepared by reaction of a biotin-water soluble linker adduct with a variety of cross-linking reagents, such as those described in the Pierce Catalog and Handbook. Homobifunctional, heterobifunctional, homotrifunctional and heterotrifunctional linkers are commercially available. For example, sulfhydryl and disulfide containing water solubilized biotin derivatives are readily available by reaction with various sulfide or disulfide containing cross-linking reagents (e.g. DTSSP, SMPT, SPDP and 2-iminothiolane).

Biotinylation reagents and biotin-containing compounds of the present invention may be linked to targeting moieties such as monoclonal antibodies, or fragments or constituents thereof. Combination of an intact monoclonal antibody, or a fragment (e.g. $F(ab')_2$, Fab', Fab, scFv, $scFv_2$) produced from biological methods, genetically engineered, and/or chemically modified, with biologically produced, genetically engineered, and/or chemically modified avidin, deglycosylated avidin, or streptavidin, forms targets for biotinylated compounds to be used in *in vitro* assays, or for diagnostic and therapeutic *in vivo* applications. An important factor in these applications is that the biotin-containing compound be water soluble, since binding proteins are most stable in aqueous media. The monoclonal antibody may be conjugated, through chemical or molecular biology techniques, with the biotin binding protein prior to its binding with the target material, or alternatively, it may be bound with the target material, then the biotin binding protein can be introduced in a second step. In this latter case, the antibody must be biotinylated using one of many different biotinylation reagents prior to targeting of the biotin-binding protein.

Water-soluble biotin-containing compounds and biotinylation reagents linked to imageable or therapeutic functional moieties can be used in *in vivo* applications for imaging and/or therapy of human disorders (e.g. cancer, blood clotting, myocardial infarcts). Example 18, for example, describes a method for preparing a water soluble biotin compound stabilized from biotinidase cleavage and having a radio-iodine reporter moiety. Water soluble biotin-containing compounds linked or linkable to radionuclides, photoactive compounds (i.e. dyes), drugs and pro-drugs, and other agents, such as boron-10 containing compounds for use in Boron Neutron Capture Therapy (BNCT), whether as

a small molecule biotin conjugate, or as a polymer or conjugate of a polymer, are described below.

Numerous diagnostic and therapeutic radionuclides can be, and have been, used with targeting moieties such as monoclonal antibodies. See, for example Srivastava, Ed., *Radiolabeled Monoclonal Antibodies for Imaging and Therapy*, 1988. Biotinylation reagents and biotin-containing compounds comprising a water soluble linker moiety may improve the properties of radionuclides that are used in combination with monoclonal antibody targeting. For example, the *p*-(radio)halobenzoyl adduct of structure **8**, illustrated below as structure **30**, can be prepared by radiohalogenation of *p*-tri-*n*-butylstannylbenzoyl derivative shown below as structure **31**. Subsequently, the arylstannane **31** can be used to prepared high specific activity radiohalogenated derivatives in which X = ^{18}F, ^{75}Br, ^{76}Br, ^{77}Br, ^{80m}Br, ^{122}I, ^{123}I, ^{124}I, ^{125}I, ^{131}I, ^{211}At. *In vitro* diagnostic tests are routinely conducted with ^{125}I. Diagnostic *in vivo* applications may be accomplished for example, using ^{77}Br, ^{123}I or ^{131}I and employing gamma cameras or SPECT instruments as detection devices. Alternatively, PET scanning may be achieved with the positron emitting radionuclides ^{18}F, ^{75}Br, ^{76}Br, and ^{124}I. Therapeutic applications may employ beta or positron emitting radionuclides, or the alpha emitting radionuclide ^{211}At.



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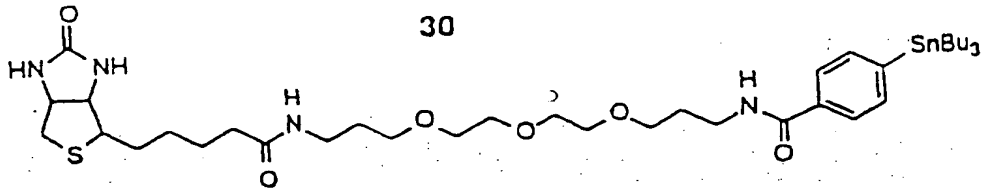

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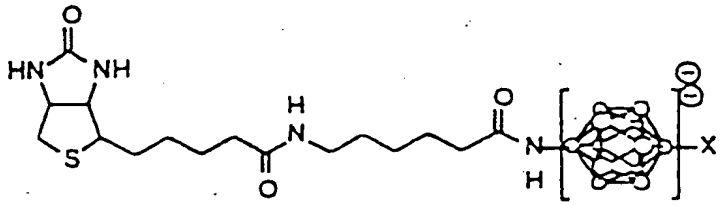
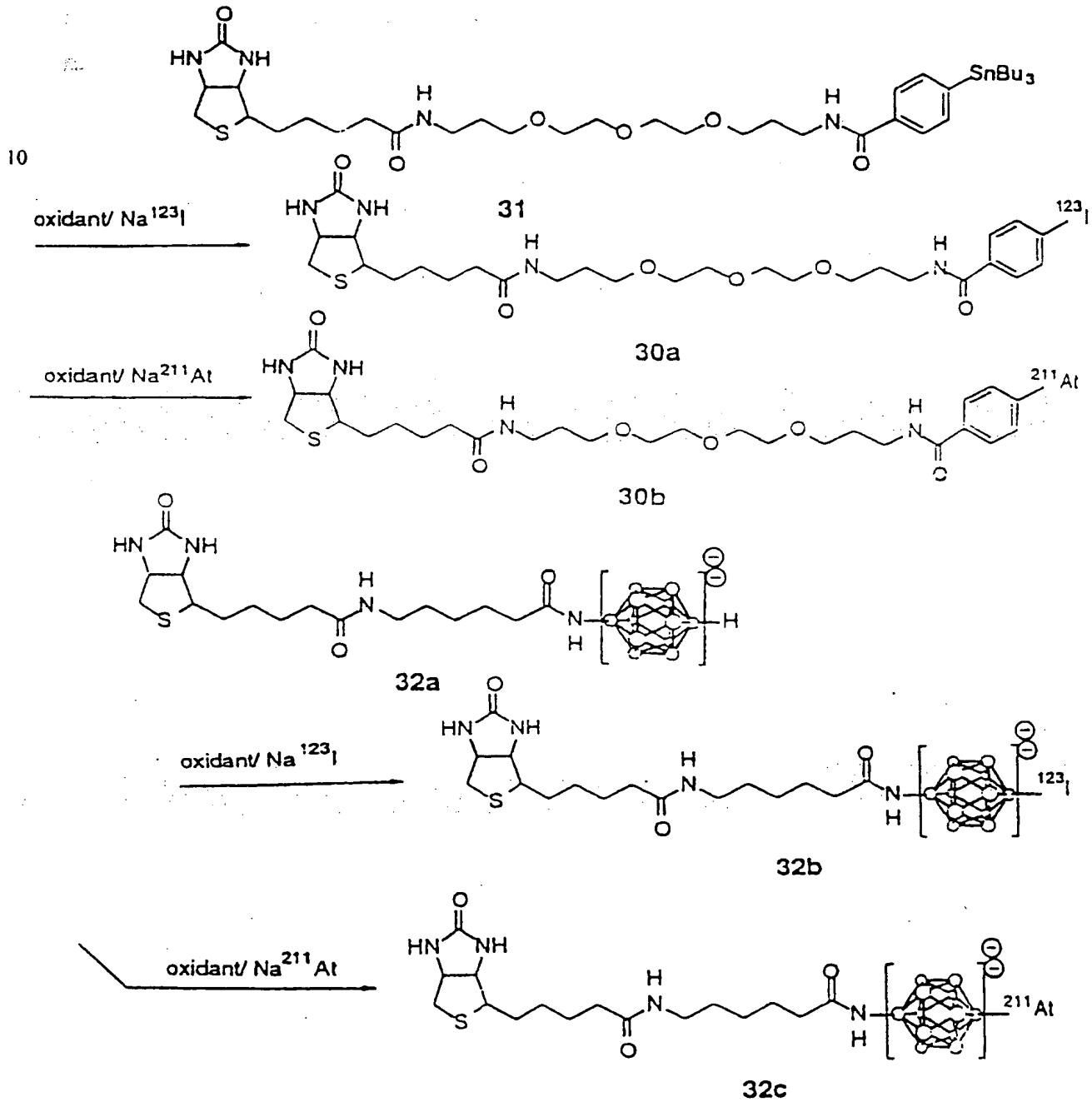


32

Radiohalogen compounds comprising ^{123}I (shown in structure **30a**), ^{211}At (shown in structure **30b**), ^{123}I (shown in structure **32b**), and ^{211}At (shown in structure **32c**), below, are particularly beneficial for *in vivo* diagnostic imaging and therapy applications. Exemplary syntheses of these compounds are illustrated below. Other linking moieties with aryl or vinyl radiohalogen derivatives can also be prepared as described in Wilbur, *Bioconjugate Chem.*, 1992.

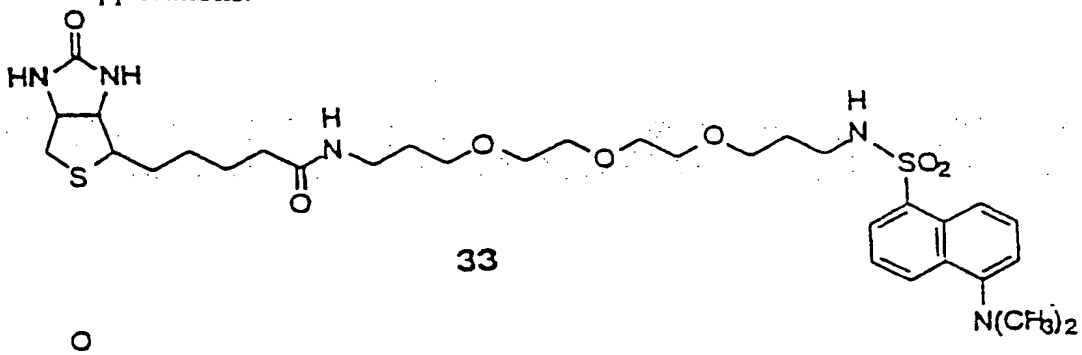


Biotinylation reagents and biotin-containing compounds that are chelatable to radionuclides other than radiohalogens also benefit from having a water soluble linker moiety between the chelate and the biotin moiety. The water soluble linker moiety provides better interaction with biotin-binding proteins. Water solubilizing biotin-chelate conjugates may be synthesized, for example, by conjugating the trioxobiotin-glycolate TFP ester illustrated in structure **24**, with ω-terminal amino derivatives of chelates known as EDTA and DTPA (described in Subramanian, *Cancer Imaging and Radiolabeled Antibodies*, pp 183-199, 1990) and cyclic chelates known as NOTA, DOTA, and TETA (described in Gansow, In *Cancer Imaging and Radiolabeled Antibodies*, pp 153-171, 1990). Such biotin-containing compounds may provide chelates for many different radionuclides, including In-111, Y-90, Ga-67, Ga-68, Cu-64, Cu-67, Sm-153, and Bi-212. In another exemplary conjugation, the amino-trioxo-biotin compound illustrated as structure **8** may be conjugated with chelates known as N_2S_2 (described in Fritzberg, *Proc. Natl. Acad. Sci. USA*, 85, 4025-4029, 1988) or N_3S (described in Gerretsen, *Cancer Res.* 53, 3524-3529, 1993) to chelate Tc-99, Tc-99m, Re-186, Re-188.

Another important aspect of the invention is the preparation of radionuclide carrying biotin-containing compounds that exhibit reduced binding affinity for the biotin-binding proteins avidin and streptavidin. Such reduced binding affinity biotin compounds are suitable for targeting a specific sites such as a tumor with a diagnostic radionuclide having reduced binding affinity to obtain information on location, size, vascularity, etc. A higher affinity biotin-containing compound conjugated to a therapeutic radionuclide can then be administered to displace the diagnostic radiolabeled biotin moiety from its target site. In this manner, similar localized antibody-biotin binding protein complexes can be used for both diagnosis and therapy.

Biotin-containing compounds comprising UV, visible, or infrared active compounds may be prepared for a variety of applications. For purposes of *in vitro* diagnostic assays, the dansyl derivative, **33**, or the fluorescein derivative, **34**, are useful. These are but two examples of a large number of imaging agents that have various adsorption and photochemical properties. Numerous other imaging agents, such as those described in Haugland, *Molecular Probes* catalog and references therein, may be

conjugated to biotinylation reagents of the present invention and utilized for *in vitro* or *in vivo* applications.



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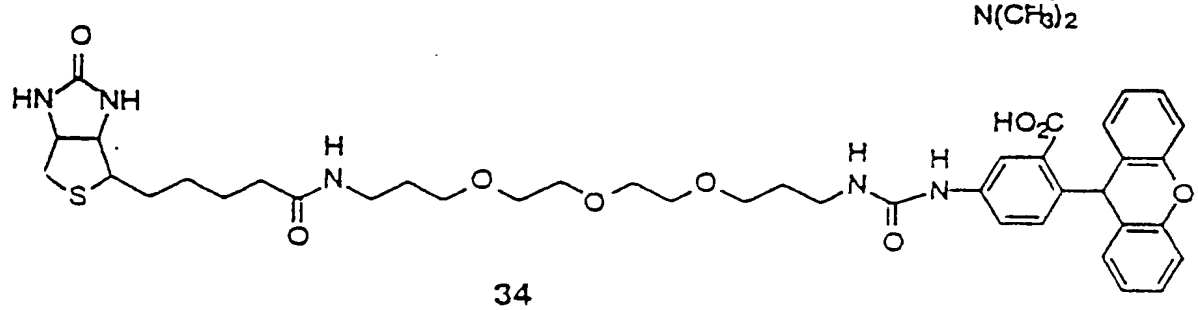
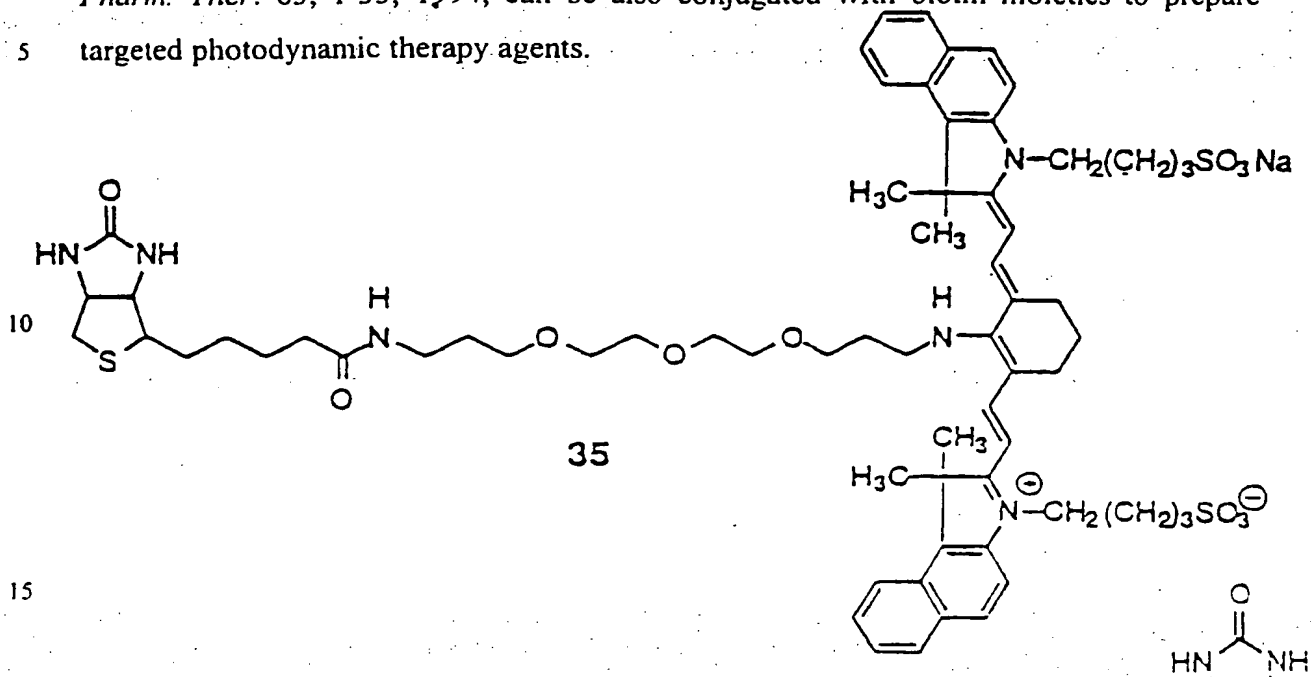
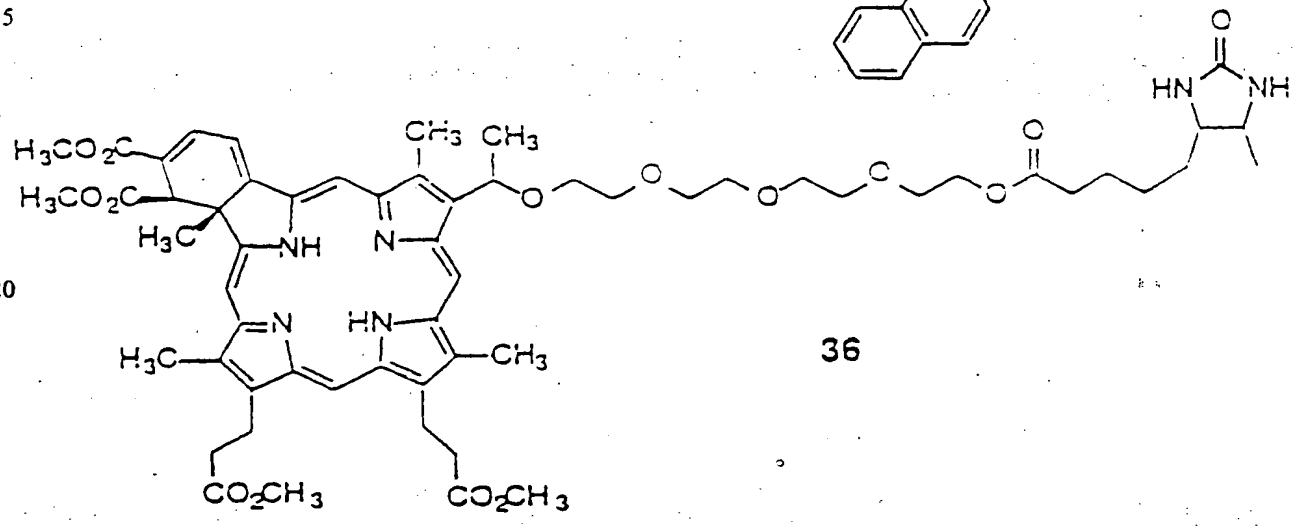


34

Biotin-dye conjugates can be used for *in vivo* detection of disorders when combined with targeting moieties such as monoclonal antibodies. To be effective at depths of more than a few millimeters, diagnostic dyes preferably have absorption maximum in the range of 550 - 1200 nm. Preferred diagnostic dyes are cyanine dyes, and a biotin conjugate of a cyanine dye is illustrated below as compound 35. Many different cyanine dye-biotin moiety conjugates can be prepared by reaction of a terminal amine containing a water soluble biotin moiety, with a cyanine dye containing a carboxylate group, or activated carboxylate group. Suitable conjugation techniques and dyes are described, for example, in Mujumdar, *Cytometry* 10, 11-19, 1989; Southwick, *Cytometry* 11, 418-430, 1990; and Mujumdar, *Bioconjugate Chem.* 4, 105-111, 1993. Water soluble biotin-containing compounds of the present invention which contain a terminal sulfhydryl group can be reacted with cyanine dyes which are appropriately derivatized, as described in Ernst, *Cytometry* 10, 3-10, 1989. Photodynamic dyes that are useful for therapeutic applications, such as photodynamic therapy ("PDT"), are described in Rosenthal, *Ann Med.* 26, 405-409, 1994. Biotinylated PDT-active dye conjugates, used in combination with targeting functions provided by monoclonal antibodies/biotin binding proteins, can improve PDT. An exemplary benzoporphyrin derivative which has a desthiobiotin

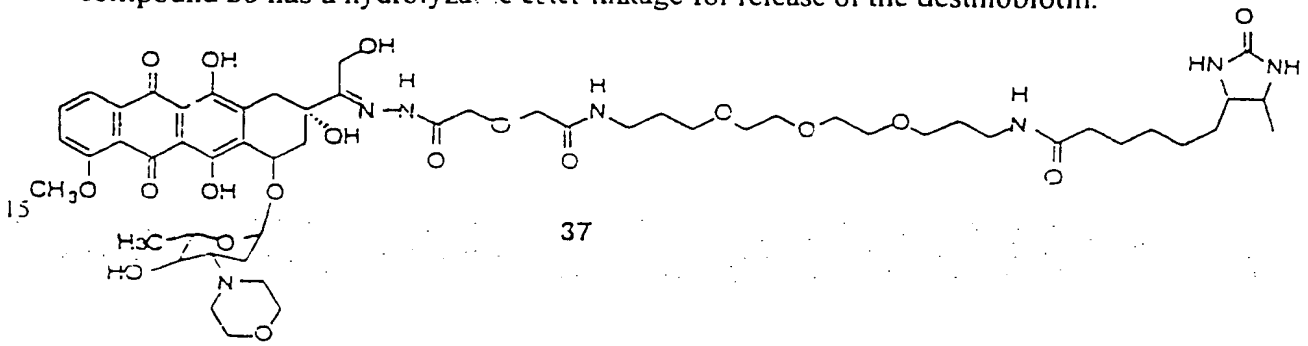
attached is illustrated as compound 36. Compound 36 has an ester linkage to the desthiobiotin so that it can be cleaved from the porphyrin prior to cellular localization. A large number of alternative photosensitizer dyes, such as those described in Diwu, *Pharm. Ther.* 63, 1-35, 1994, can be also conjugated with biotin moieties to prepare targeted photodynamic therapy agents.





Another application for water soluble biotin-containing compounds and biotinylation reagents is to provide a targeting system that can be used with therapeutic drugs. Targeting of any number of therapeutic drugs to sites such as tumors can be accomplished with this system, and biotinylated therapeutic drugs can be released at a selected site. However, it is also important in many instances to release the biotin from the drug such that its most active form is in the cell. This may be accomplished by

introducing one or more cleavable functional groups at the point of attachment of the drug. For example, a morpholino-doxorubicin (See, Mueller, *Antibody, Immunoconjugates, Radiopharm.* 4, 99-106, 1991) adduct of desthiobiotin is illustrated as compound **37**. Additionally, an example of a vinca alkaloid conjugate (Laguzza, *J. Med. Chem.* 32, 548-555, 1989) of desthiobiotin is illustrated as compound **38**. Both of these examples are designed to be released from the antibody-biotin binding protein complex at a preselected site, such as a tumor. Release may be brought about by biotin in the diet (slow release), or it may be brought about by introduction of biotin by other means such as osmotic pump, injection, or the like. Compound **37** has a hydrolyzable hydrazide linkage to the desthiobiotin for its release in the cell (or in acid tumor environment), and compound **38** has a hydrolyzable ester linkage for release of the desthiobiotin.



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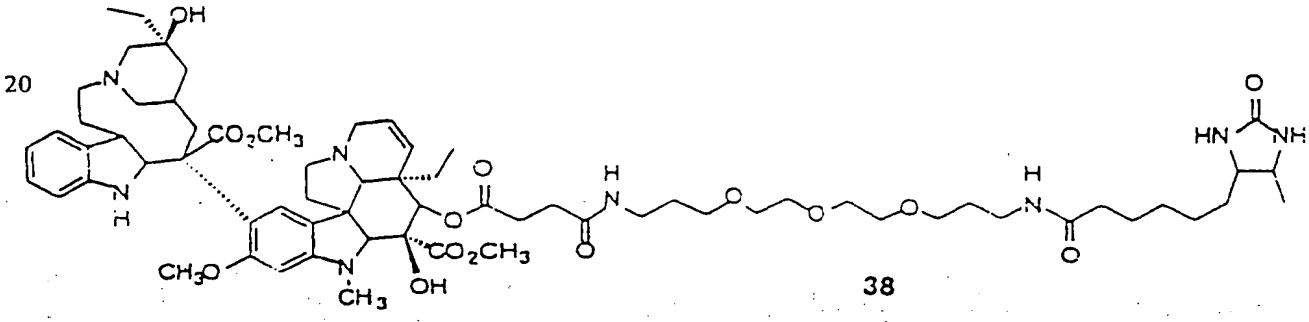
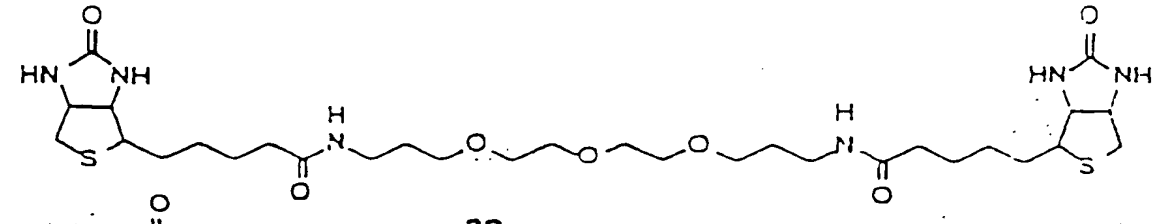


38

Another application of water solubilized biotin-containing compounds and biotinylation reagents is to provide a targeting system that can be used with boron-10 containing compounds. Targeting of any number of boron-10 containing compounds, such as small molecules or polymers, to preselected sites such as tumors can be

accomplished with this system. A preferred example of a biotin-boron-10-containing polymer is prepared from a starburst and cascade dendrimer (See, Tomalia, In *Topics in Current Chemistry*, 165, 193-313, 1993), where 1-20 biotins (depending on the size of polymer) are first conjugated with the dendrimer, then 10-200 boron-10 containing cage molecules (e.g. *closo*- or *nido*-borane, carbaborane, or dicarbaborane; Hawthorne, *Angew. Chem. Int. Ed. Engl.* 32, 950-984, 1993; Morin, *Tetrahedron* 50, 12521-12569, 1994) are conjugated. The resulting biotinylated boron-10 containing polymer may be used with prelocalized monoclonal antibody/biotin binding proteins to localize boron-10 to selected sites, such as tumors for subsequent neutron irradiation in a therapeutic protocol.

Dimeric, trimeric, and other multimeric biotin compounds can be prepared for cross-linking of the biotin-binding proteins. Biotin dimers, trimers and multimers made using hydrophobic linker moieties are highly insoluble in aqueous solutions, which renders them unusable for *in vivo* applications. One application for polymerization of biotin-binding proteins is clearance of the antibody-biotin binding protein, or non-bound biotin-binding protein, from the blood of patients which are involved in procedures where targeting of tumor sites in the body is achieved with monoclonal antibodies conjugated with biotin-binding proteins. A few examples of the many possible dimeric biotin-containing compounds comprising water solubilizing linkers are illustrated below as compounds **39 - 47**.



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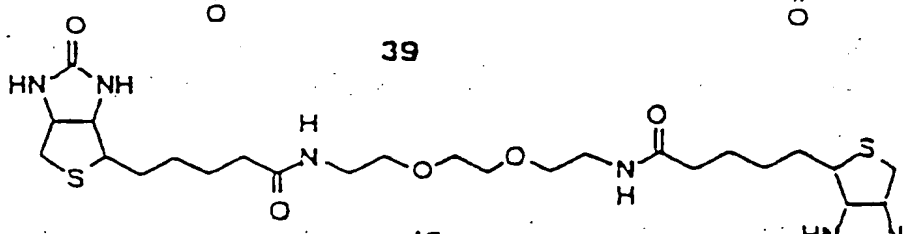

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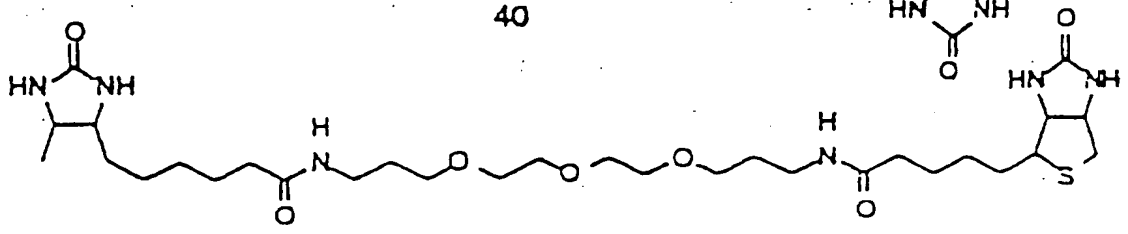

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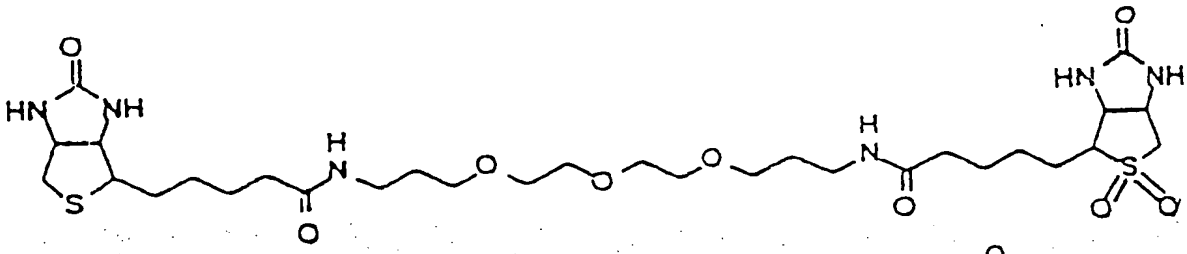

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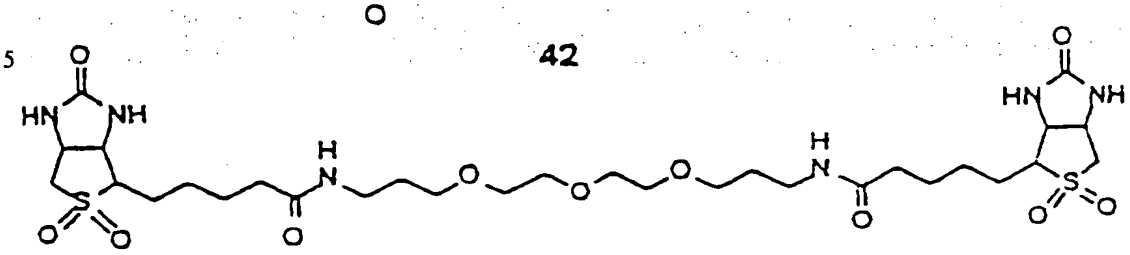

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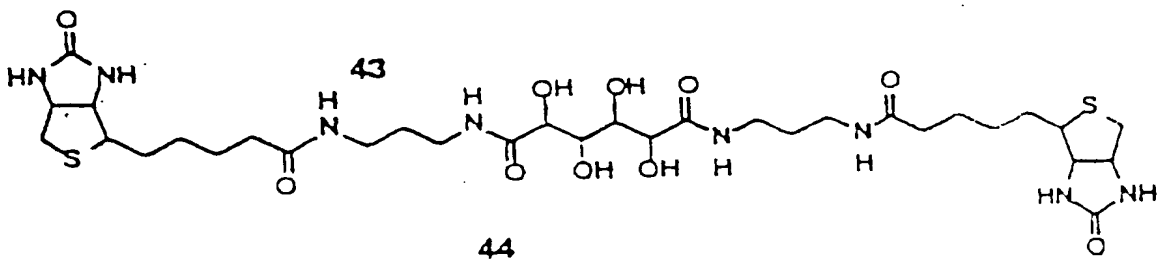

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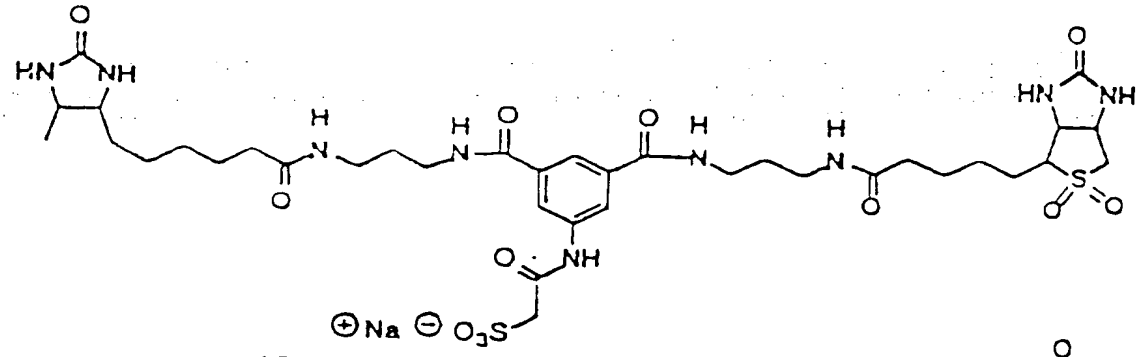

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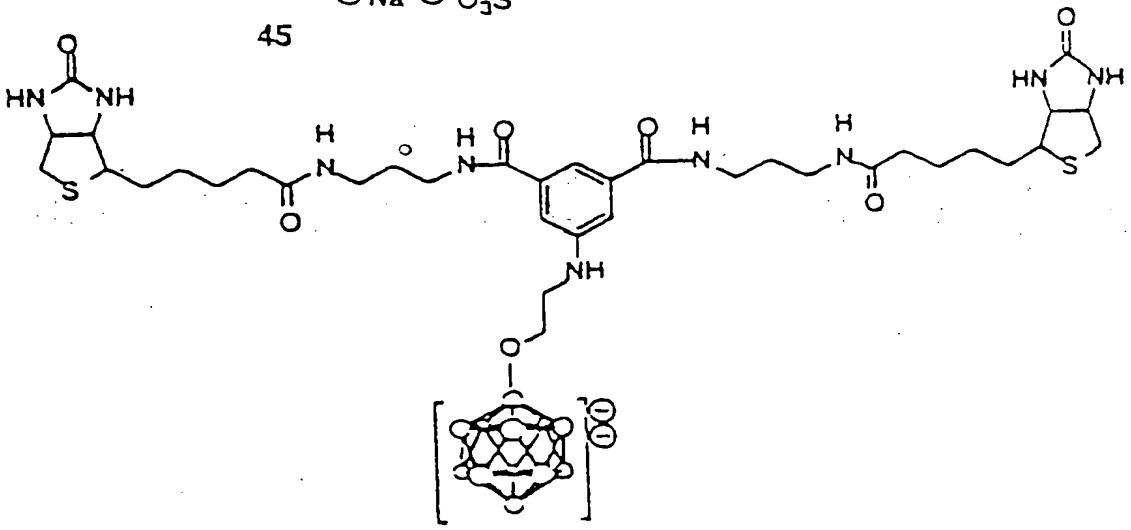
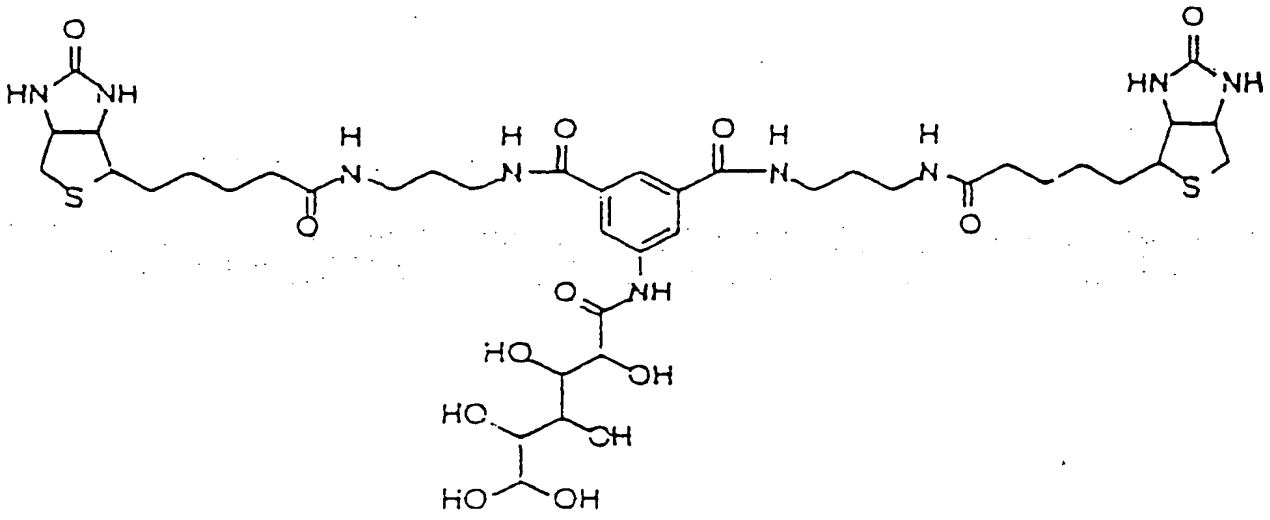


46

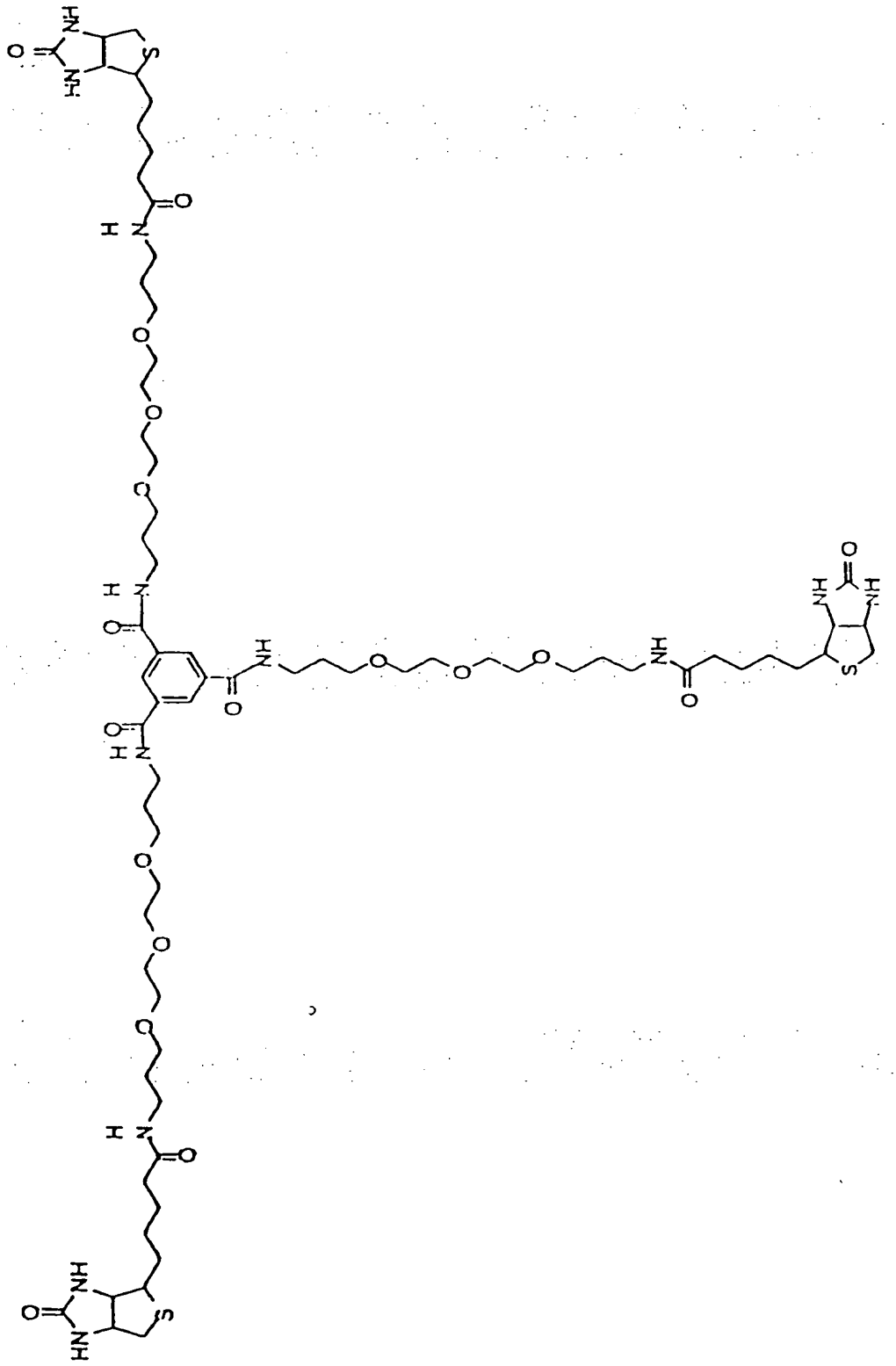


47

The compounds illustrated above are considerably more water soluble than their aliphatic counterparts. While biotin dimers linked by aliphatic linker moieties are substantially insoluble, the biotin dimer illustrated as compound 39 has a solubility of about 9 mg/mL in water at ambient temperature. Importantly, the distances between the two biotin moieties are long enough (> 15 Å) to bind two proteins, but short enough (< 20 Å) such that the two biotins will not bind to the same avidin or streptavidin molecule. Pre-incubation of any of the dimeric biotin derivatives to saturate the biotin binding sites without polymerization may be used to increase the biological half-life of the biotins, and may be used to prepare mixed polymers, wherein, the saturated biotin binding protein can polymerize in a 1:1 ratio with another biotin binding protein (as a tetramer) (e.g. ...avidin-streptavidin-avidin-streptavidin-avidin...).

Trimeric biotin compounds may also be prepared for cross-linking of biotin-binding proteins. Compounds **48-51**, illustrated below, are exemplary of trimeric biotin compounds comprising water soluble linkers. In these compounds, the biotin moieties are at a distance from one another that permits two of the biotin moieties to bind with one tetrameric biotin binding molecule, while the third biotin moiety will be free because it does not have a linker of sufficient length to bind at a third site on the same protein molecule. The distance between each biotin moiety in a biotin trimer is preferably from about 20 to about 50Å. In compound **50**, the free biotin moiety is the weaker binding

desthiobiotin, and this can result in preparing polymers which are unstable. However, unstable polymers can also be cleared from the blood, which is desirable under some circumstances. Biotin trimer **51** has two desthiobiotin moieties. In this example, four of the trimeric biotin molecules can combine with one (tetrameric) avidin or streptavidin molecule. This permits more (total of 8) desthiobiotin moieties on the molecule, and can lead to branching in the polymers formed.



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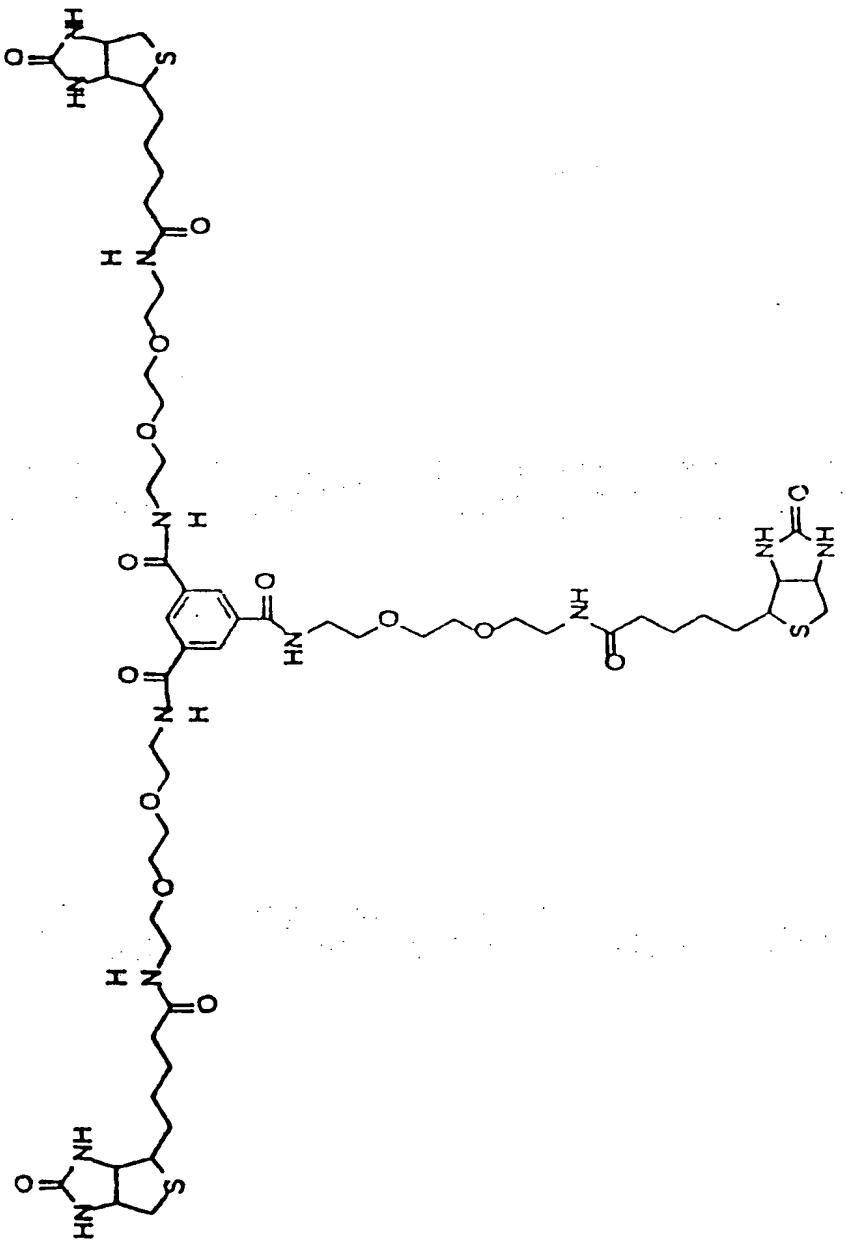

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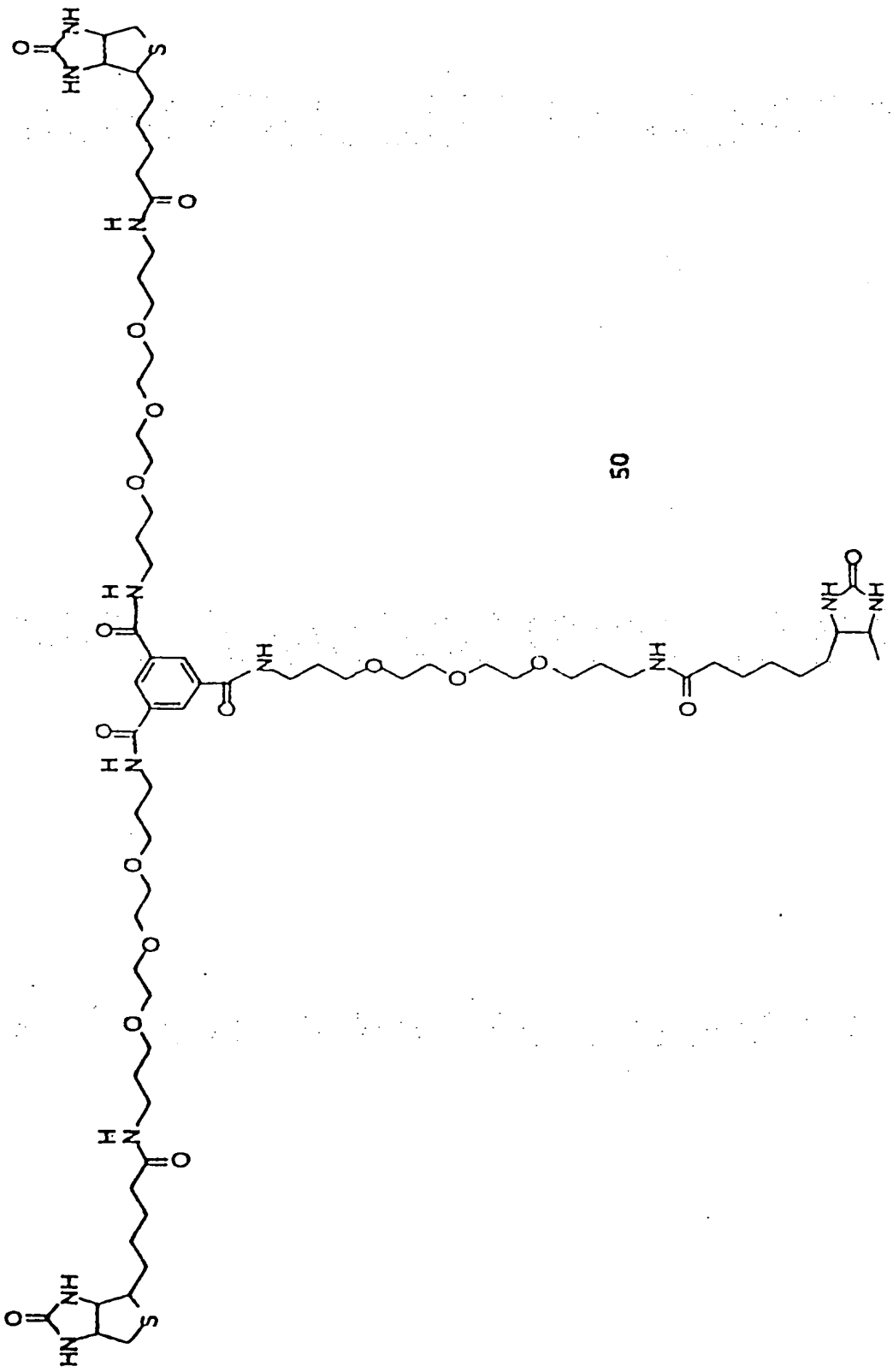

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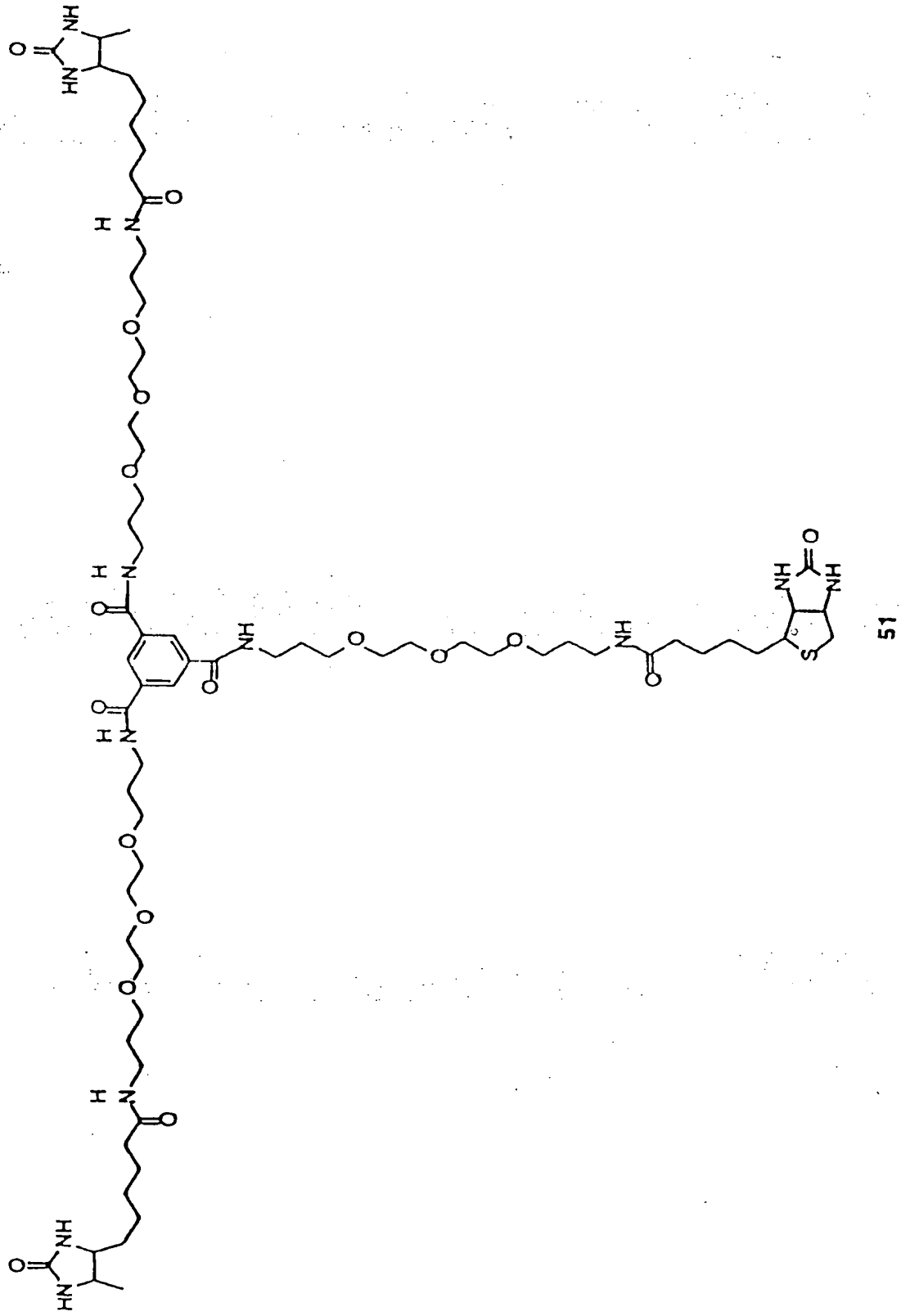


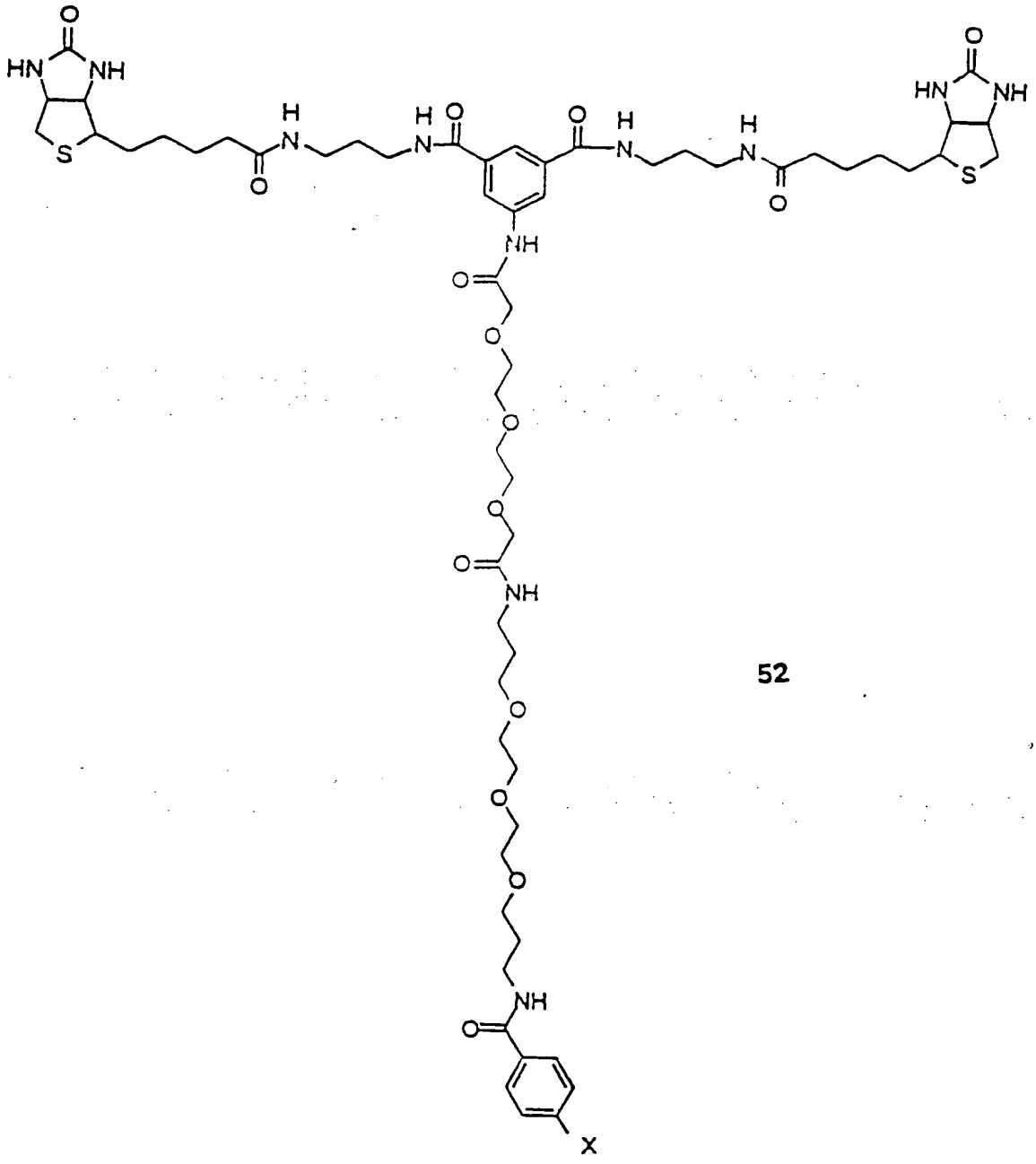
51

Biotin multimers having more than three biotin moieties are useful for blood clearance of biotin binding proteins, and are also useful for other applications such as amplification of signals in diagnostic or therapeutic systems. Multimers in which the biotin moieties are joined by water soluble linker moieties demonstrate enhanced water solubility. A preferred series of compounds which are biotinylated with multiple biotins are known as starburst and cascade dendrimers (See Tomalia, In *Topics in Current Chemistry*, 165, 193-313, 1993). For example, reaction of a terminal amine containing starburst dendrimer (e.g. generation 2, available from commercial sources) with the activated carboxyl-biotin derivative **24** produces a compound which has up to 16 biotin molecules attached. Attachment of multiple water solubilized biotin derivatives to proteins is achieved through the use of biotinylation reagents such as those shown in compounds **24-29**.

Dimeric, trimeric and multimeric biotin compounds comprising other functional moieties such as radionuclides, photoactive groups, or drugs preferably incorporate water soluble linker moieties. Such dimeric, trimeric and multimeric compounds are of interest in polymerizing biotin-binding proteins. An important application of these compounds is to increase the amount of radioactivity, photoactive moiety, or drug at a preselected site, such as a tumor, by introducing new biotin sites for biotin-binding proteins to bind to. Thus, methods for amplifying the number of sites for binding biotin-binding proteins at a preselected target, such as a tumor, would involve multiple alternating administrations of a biotin trimer or the like, and a binding protein, such as avidin or streptavidin. A functional reporter moiety such as a targeting, diagnostic, or therapeutic agent, or the like may be linked to the biotin compound or binding protein compound. When this is accomplished in a set of steps, an amplification of the amount of sites available at the tumor is obtained, since upon each avidin or streptavidin binding to a site, additional biotin binding sites are provided. This amplification can be accomplished *in vitro* as well to improve detection of samples. Successful amplification was accomplished experimentally using trimeric biotin compounds, as described below in Example 14.

An exemplary biotin dimer which can cross-link two streptavidin or avidin molecules while having a radiohalogen (X as previously defined) attached is illustrated as compound **52**. The corresponding arylstannane is illustrated below as compound **53**. It is

important to note that the water solubilizing linkers cannot be attached to the biotin moieties in these compounds as that would lead to the two biotins being of a distance apart that would result in both biotins binding to the same biotin-binding protein, which would not lead to cross-linking or amplification. Therefore, two water solubilizing moieties are combined in the molecule in a location that is unimportant in the binding process. A water soluble (ionic) biotin dimer which does not have the long ether chain is shown as compound **46**. Radiohalogenation of **46** provides **54**.



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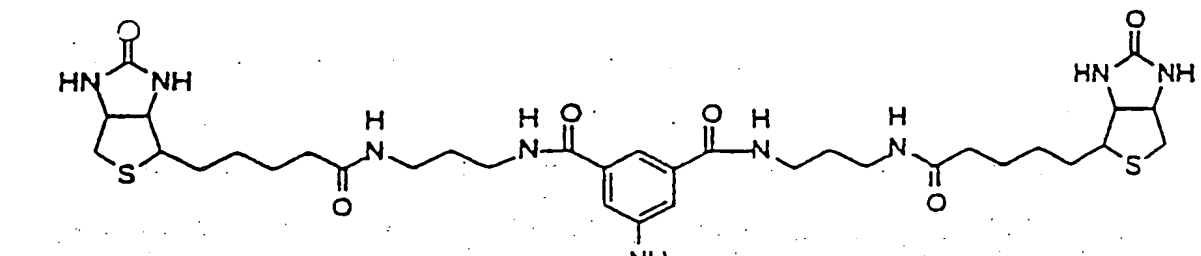
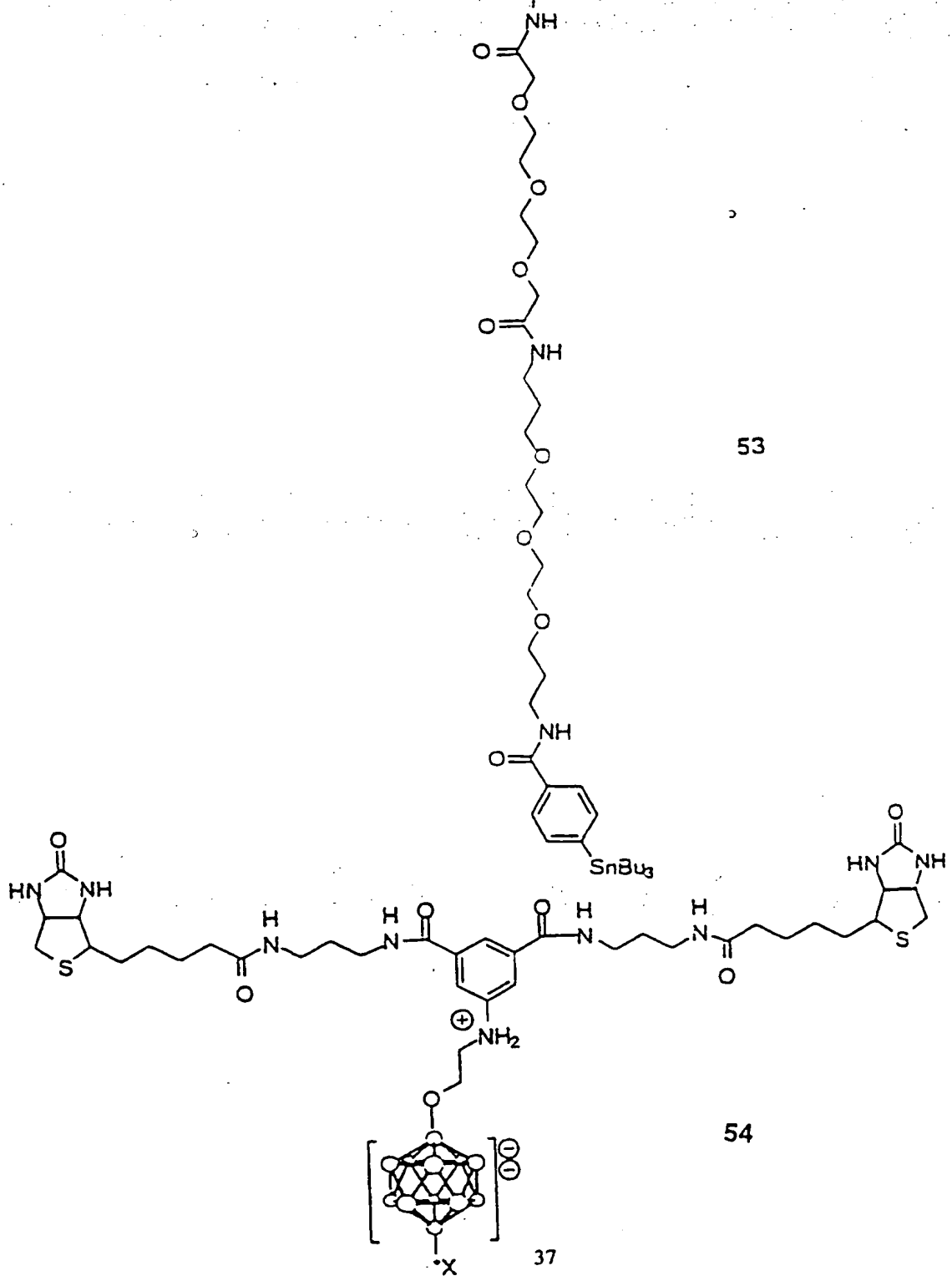
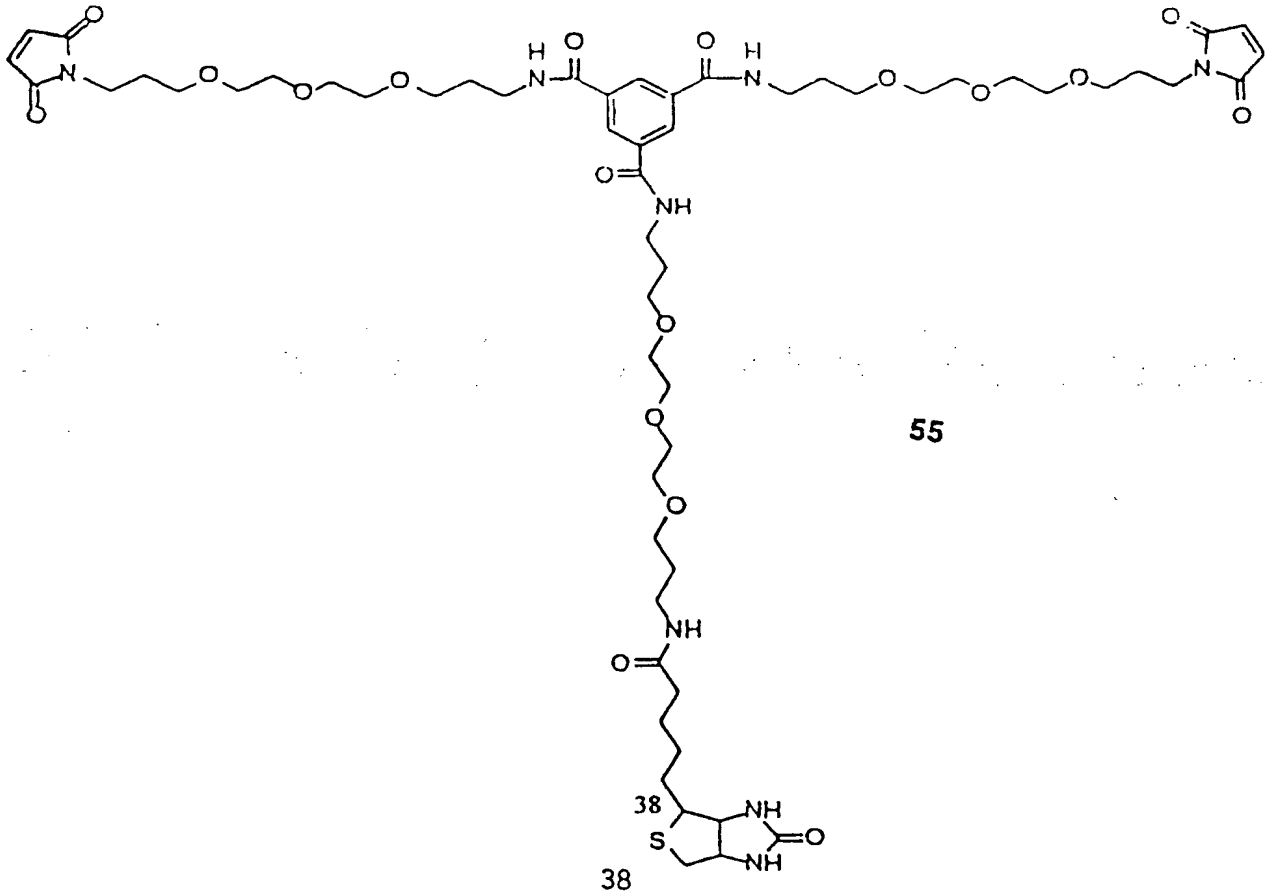


53



54

Water soluble biotinylation reagents can be prepared comprising a biotin moiety, a functionality for conjugation (e.g. amine, carboxylate ester, maleimide, acyl hydrazine, alkoxylamine) and another functionality. These trifunctional reagents have many applications, which are made possible by the water solubility conferred by the water soluble linker moiety. The linker moieties also provide for a distance of separation of the molecule such that they can be conjugated more efficiently. An exemplary reagent is illustrated as compound **55**, wherein the biotin-containing trifunctional reagent also has two maleimide groups. This compound may be used to cross-link two other compounds. It is particularly advantageous for cross-linking antibody Fab' molecules to form site-specifically biotinylated $F(ab')_2$ molecules. Such $F(ab')_2$ molecules have application in the methods of tumor targeting already presented herein. Reactive groups other than maleimides may also be used for cross-linking. Another trifunctional biotin-containing reagent is shown in compound **56**. In that compound there is a biotin, a conjugation group (maleimide) and a radiohalogenated moiety (X as previously defined). These water soluble biotinylation reagents, compounds, and many other combinations of biotin containing trifunctional reagents can be prepared for diagnostic or therapeutic applications.



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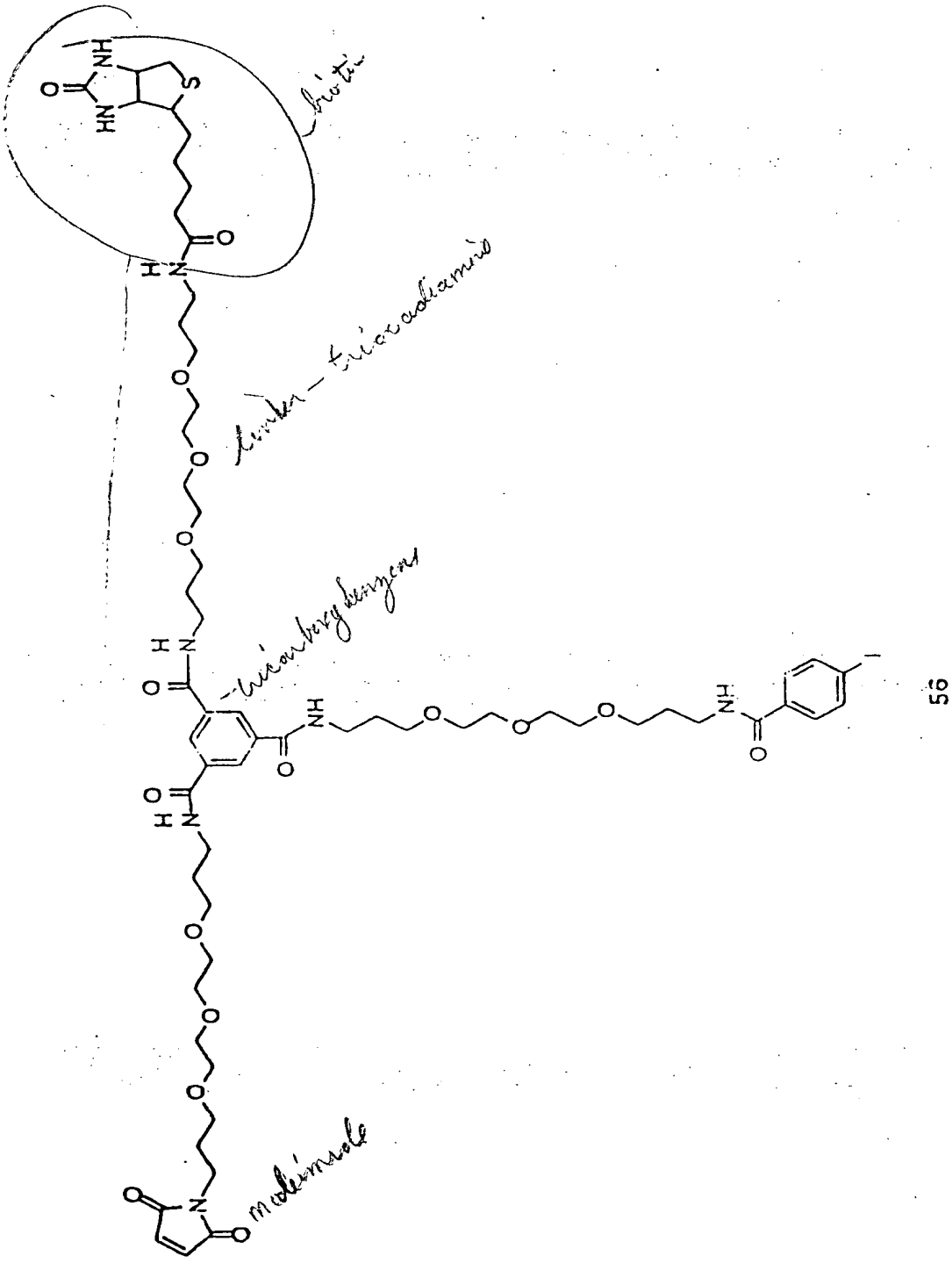
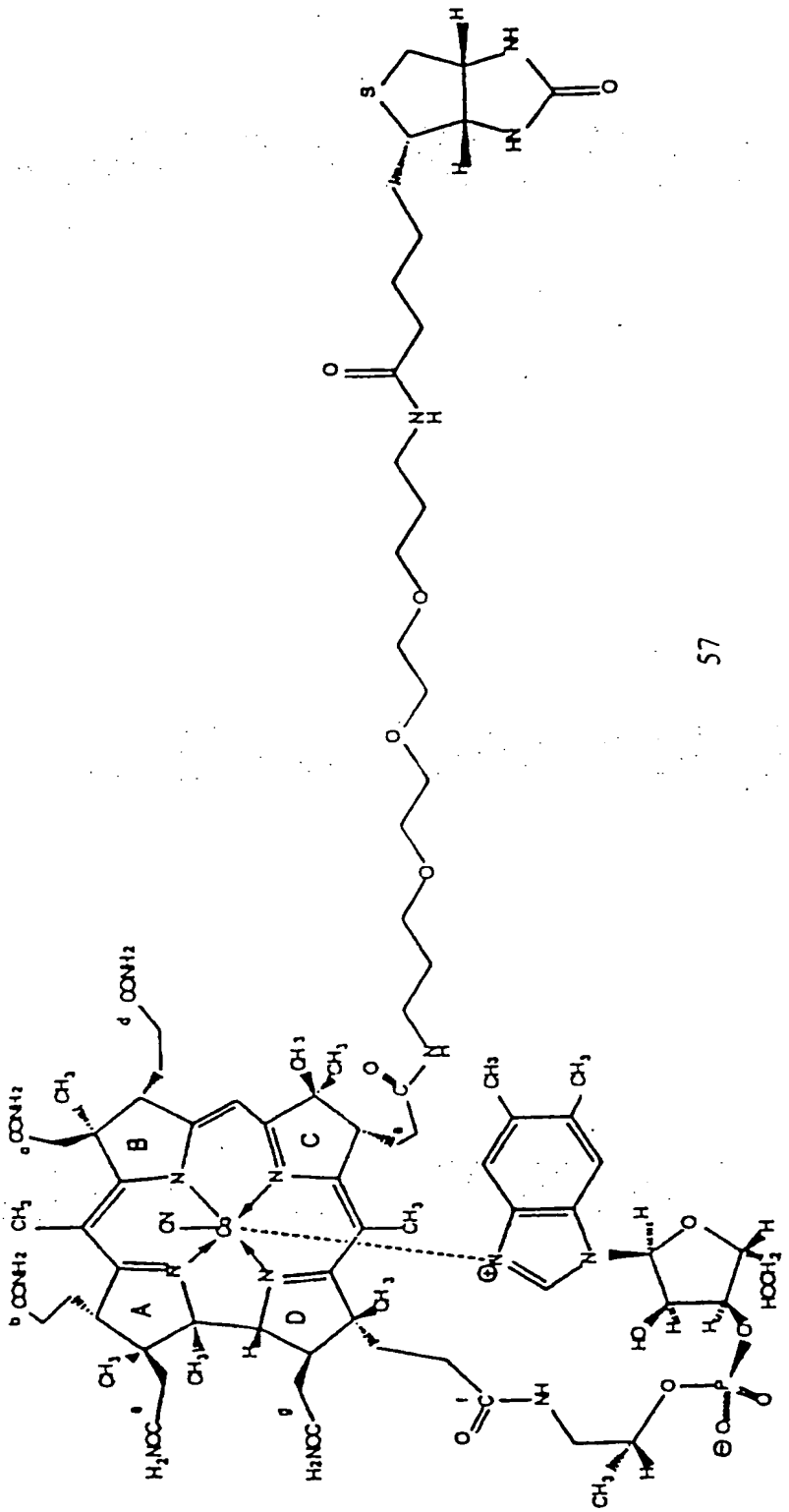

biotin
linker – tricarboxamide
tricarboxybenzene
maleimide
56

Water soluble linker moieties that provide a desired distance between an avidin or streptavidin binding biotin moiety and another moiety that binds to a specific carrier or receptor protein improve such compounds. Water solubility is necessary for the protein interaction, but the water soluble linker moiety also permits the biotin and other molecule to extend from one another in aqueous media such that binding with two different proteins can be achieved. This is particularly important to have an availability of the compound to bind a second protein once the first protein has been bound, and where the protein binding molecule is sensitive to steric encumbrance to its binding pocket. An example of such a protein binding molecule is the cyanocobalamin conjugate of biotin, illustrated below as structure 57. Compound 57 was found to be much more water soluble than the corresponding 1,12-diaminododecane linked biotin and cyanocobalamin. The improved water solubility of biotin compound 57 over the alkyl counterpart made this compound much more useful. This was also true for the dimeric cyanocobalamin derivative shown as compound 58. The combination of other specific protein binding molecules such as hormones (e.g. steroids), neurologic binding molecules, such as those that bind to dopamine or seratonin receptors, peptides that bind to receptors (e.g. somatastatin), and the like with biotin with the water solubilizing linkers improve their properties as well.



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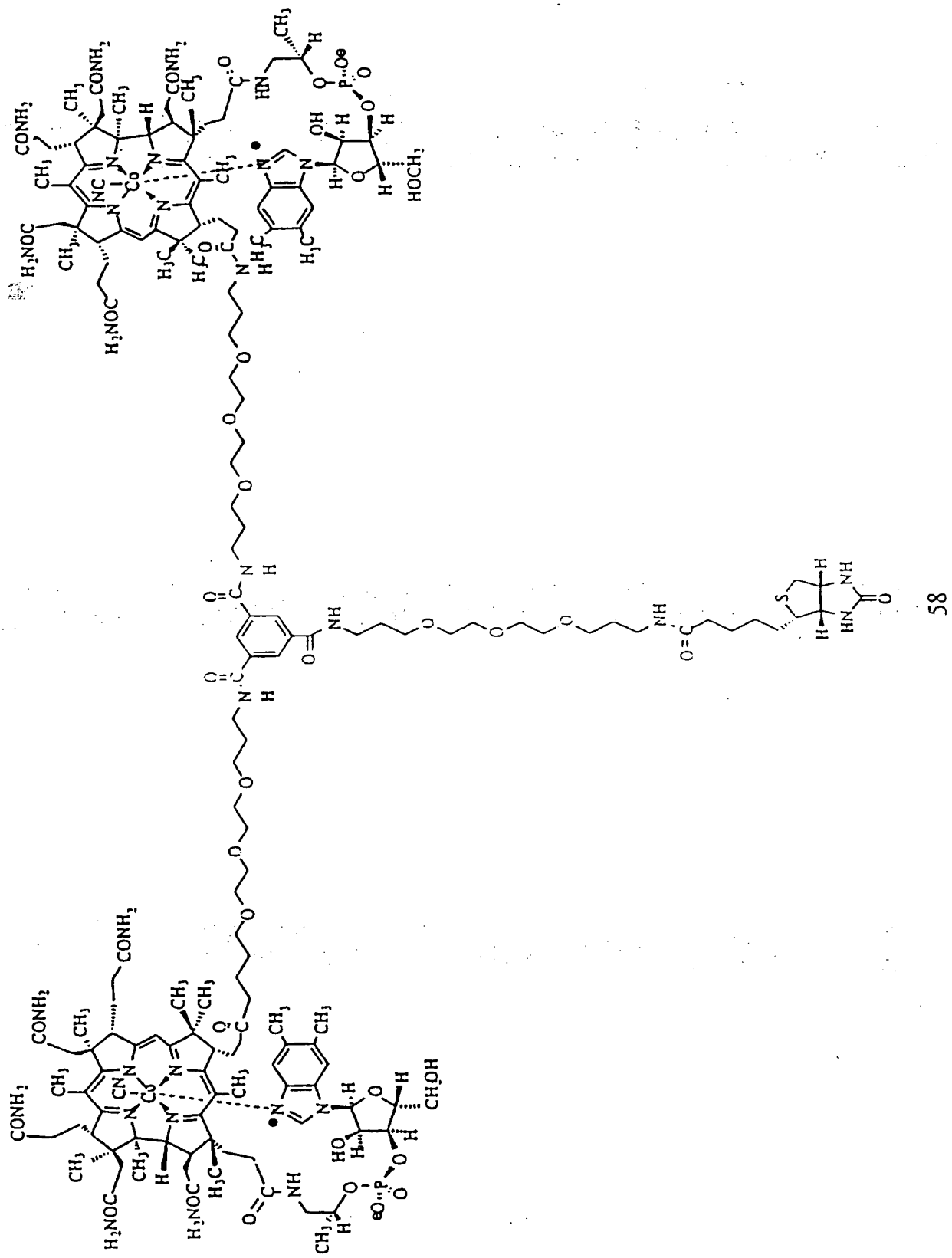


58

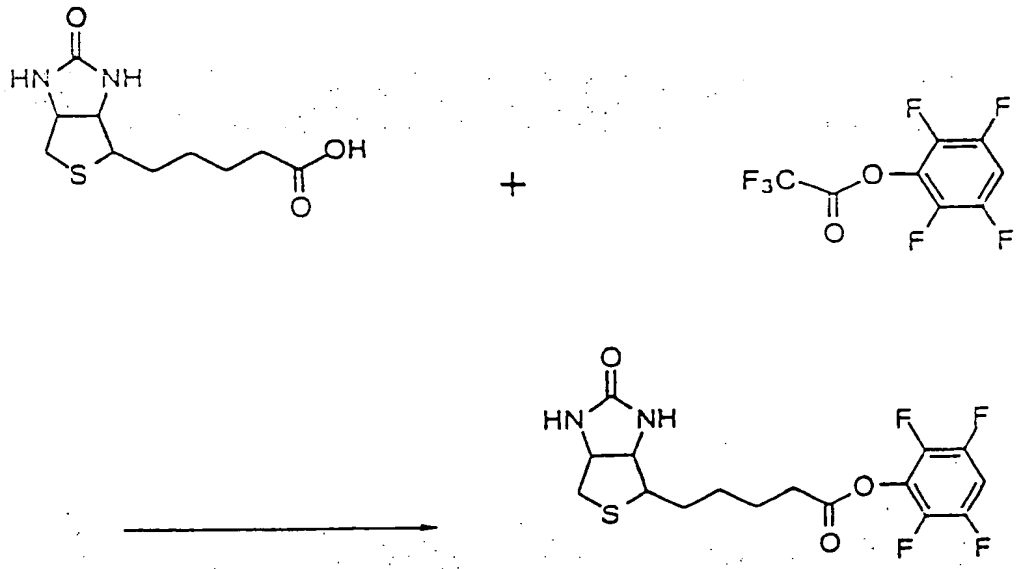
EXAMPLES

The following examples demonstrate methods for synthesizing or demonstrating the utility of various compounds disclosed herein. The examples are provided by way of illustration, and not by way of limitation.

Example 1

Method for Synthesizing an Activated Ester of a Biotin Moiety

This example sets forth a methodology for preparing a preferred tetrafluorophenyl (TFP) ester of a biotin moiety. Other methods for preparing activated esters generally known in the field can be used to prepare biotin activated esters containing any number of phenolic and other hydroxyl (e.g. N-hydroxylsuccinimide) groups.



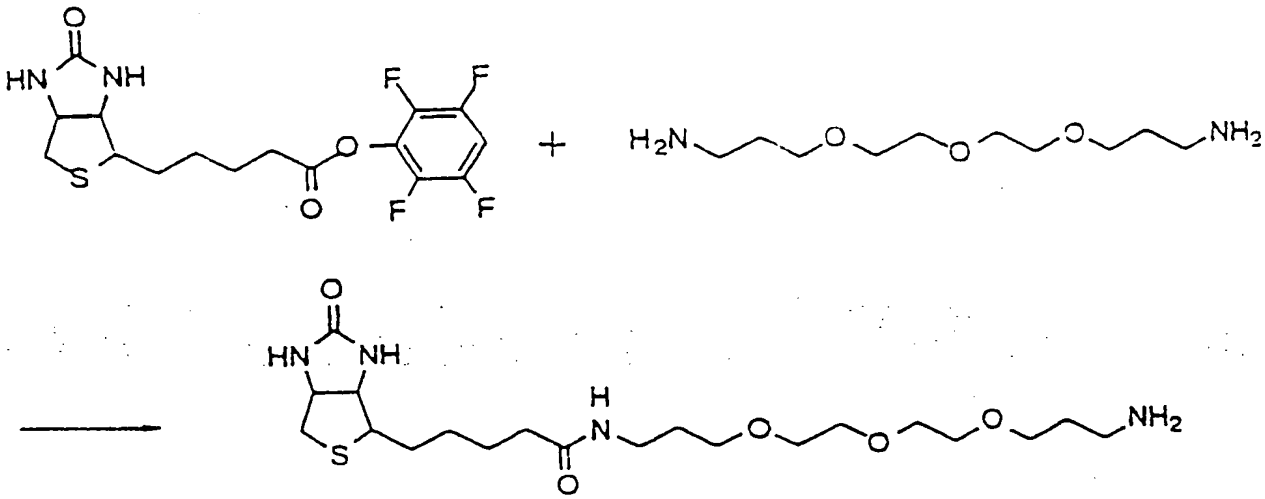
Preparation of biotin tetrafluorophenyl ester: Biotin (10 g, 40.9 mmol) was dissolved in 200 mL warm (70°C) DMF under argon atmosphere. The solution was allowed to cool to ambient temperature, and 10 mL (82 mmol) triethylamine was added, followed by the addition of 16 g (61 mmol) 2,3,5,6-tetrafluorophenyl trifluoroacetate. The reaction was stirred at room temperature for 30 min and solvent was removed under vacuum. The

product was triturated in 100 mL ether and was filtered. The isolated product was dried under vacuum to yield 14 g (83%) of biotin TFP ester as a colorless solid, mp 185-187°C. ^{1}H NMR (DMSO-d_6, δ): 1.4-1.8 (m, 6H), 2.5 (m, 1H), 2.6-2.9 (m, 3H), 3.1 (m, 1H), 4.2 (m, 1H), 6.4 (d, 2H), 7.9 (m, 1 H); IR (KBr, cm^{-1}) 3250, 2915, 1790, 1710, 1520, 1480, 1090.

Example 2

Method for Preparation of a Biotin Moiety Conjugate of a Linker Molecule

This example sets forth a general methodology for preparing a biotin moiety linked to a soluble linker moiety. In the example, a diamino-ether linker is used. Ether linkers containing terminal functionalities such as: an amine and a carboxylate; an amine and an alcohol; an alcohol and a carboxylate; and two alcohols, can also be prepared using the method described. The general method can also be used when the linker contains polyhydroxyl groups if the terminal functionalities are two amines, or are an amine and a carboxylate.



Preparation of a biotin compound containing a trioxo-amide linker with an amine terminus: TFP ester of biotin (5 g, 12.8 mmol) was added in a dry flask and was dissolved in 200 mL anhydrous DMF. In another dry flask containing 28 g, 128 mmol)

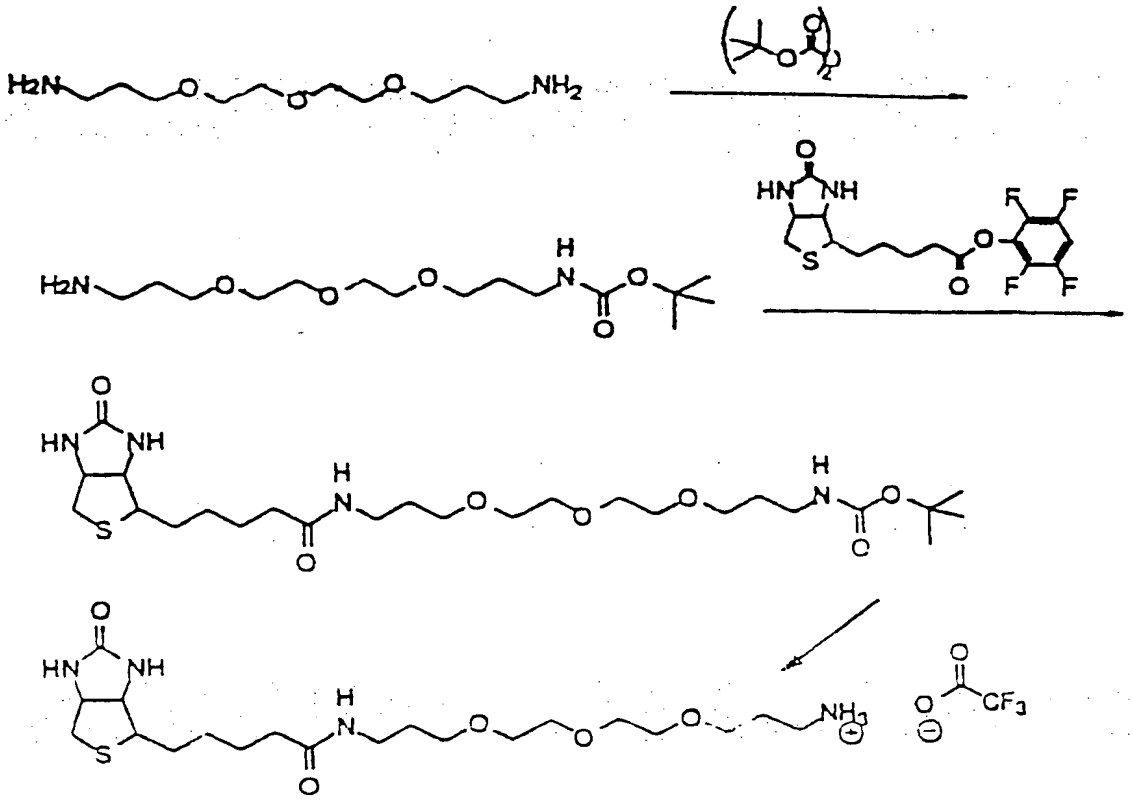
4,7,10-trioxa-1,13-tridecanediamine was added 4 mL of triethylamine. Both the flasks were cooled to 0-5°C by ice water. The TFP ester of Biotin was added dropwise to the trioxatridecanediamine solution over the period of 1 h. The reaction was stirred at room temperature for 30 min and solvent was removed under vacuum. The resulting oil was triturated in 500 mL ether and was stirred for 30 min. The solid was filtered. The solid product was dissolved in methanol : ethyl acetate (8:2) and loaded on silica column. The column was eluted with methanol : ethyl acetate (8:2). Fractions containing product were collected, and solvent was removed under vacuum. The isolated product was dried under vacuum to yield 4.5 g (79%) of desired product as a colorless solid, mp 104-106°C. ^{1}H NMR (MeOH, δ): 1.46 (m, 2H), 1.6-1.8 (m, 9H), 2.2 (t, 2H), 2.7 (d, 1H), 2.75 - 2.9 (m, 3H), 3.2-3.3 (m, 5H), 3.5-3.6 (m, 14H), 4.3 (m, 1H), 4.5 (m, 1H); IR (KBr, cm^{-1}): 3280, 2910, 2850, 1690, 1640, 1110, 940.

Example 3

Method for Reacting an Activated Ester of a Biotin Moiety with a Diamino-linker That has One Amine Protected

This example sets forth a general methodology for preparing a biotin moiety linker adduct comprising a diamino-linker molecule, in which the linker has one amine group protected. In the example provided a diamino-ether linker is protected as a *t*-BOC derivative prior to addition to the biotin moiety active ester molecule. Any number of amine protecting groups can be utilized in this reaction. The primary purpose of the reaction method (versus example 2) is to be able to react one equivalent of diamino linker molecule with one equivalent of the biotin active ester.

Alternate synthesis of biotin conjugated with a trioxo-amide linker with an amine terminus



Reaction Step 1: Preparation of N-BOC-4,7,10-trioxa-tridecane-13-amine: To a solution of 151.40 g (687.25 mmol) of 4,7,10-Trioxa-1,13-tridecaneamine in 700 mL $CHCl_3$ was added 6.00 g (27.5 mmol) of di-tert-butyl dicarbonate in 100 mL $CHCl_3$ with stirring at ambient temperature over 30 min. The mixture was stirred for 12 h, washed with water (8 x 100 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give 8.23 g (25.7 mmol) of the desired product as a clear oil: ^1H NMR ($CDCl_3$, δ) 5.18 (s, 1H, CONH), 3.55 (m, 12H -OCH_2), 3.20 (m, 2H CH_2NHCO), 2.68 (t, 2H CH_2NH_2), 1.73 (m, 4H), 1.41 (s, 9H).

Reaction Step 2: Preparation of N-(13-N-BOC-4,7,10-trioxa-tridecanyl)biotinamide: To a solution containing 1.00 (2.54 mmol) 2,3,5,6-tetrafluorophenol ester of biotin in 100 mL CH_3CN at 55 °C was added 0.80 g (2.49 mmol) N-BOC-4,7,10-trioxa-13-tridecaneamine in 25 mL CH_3CN. The mixture was

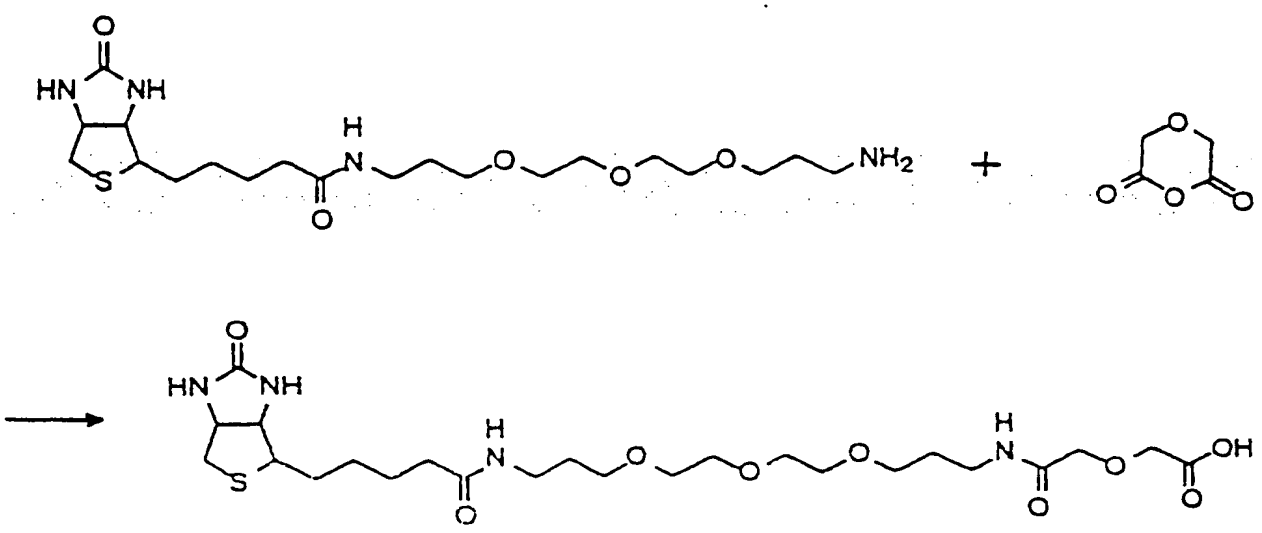
allowed to cool to ambient temperature and was stirred for 10 h. The solvent was evaporated under reduced pressure and the residue was redissolved in 200 mL EtOAc. The EtOAc solution was washed with (2 x 100 mL) 10% solution of $NaHCO_3$, (2 x 50 mL) water, dried over anhydrous Na_2SO_4 and concentrated to give the desired product as a tacky solid. ^1H NMR ($CDCl_3$, δ) 6.71 (s, NH, 1H), 6.53 (s, NH, 1H), 5.89 (s, NH, 1H), 5.06 (s, NH, 1H), 4.47 (m, 1H), 4.27 (m, 1H), 3.57 (m, 12H), 3.30 (m, 2H), 3.17 (m, 2H), 3.11 (m, 1H), 2.87 (dd, J = 12.8, 4.5 Hz, 1H), 2.71 (d, J = 12.8 Hz, 1H), 2.15 (t, 2H), 1.72 (m, 10H), 1.40 (s, 9H).

Reaction Step 3: Preparation of 13-N-Biotinamide-4,7,10-trioxa-tridecaneamine trifluoroacetic acid salt: N-BOC-trioxa-biotinamide, 1.25 g (2.28 mmol), was dissolved in 10 mL trifluoroacetic acid and stirred for 30 min at ambient temperature. Excess trifluoroacetic acid was evaporated under reduced pressure to give 1.24 g (2.17 mmol) of the desired trifluoroacetate salt as an oil: ^1H NMR (CD_3OD, δ) 4.25 (m, 1H), 4.13 (m, 1H), 3.35 (m, 10H), 3.32 (m, 2H)), 2.97 (m,4H), 2.81 (m, 2H), 2.65 (dd, J = 12.8, 4.8 Hz), 2.63 (d, J = 12.8 Hz), 1.93 (m, 2H), 1.64 (m, 2H), 1.48 (m, 4H), 1.37 (m, 2H), 1.18 (m, 2H).

Example 4

Method for Preparing a Carboxy Derivative of a Biotin Moiety Comprising a Water Soluble Linker and Glycolic acid

This example sets forth a general methodology for preparing a biotin compound comprising an w-terminal carboxylate. The method described can be applied in a general way to prepare biotin moieties with any number of linker molecules attached. While in the example described a diamino-ether linker is used, examples wherein the linker has an amine and an alcohol at the termini are also provided for with this method.

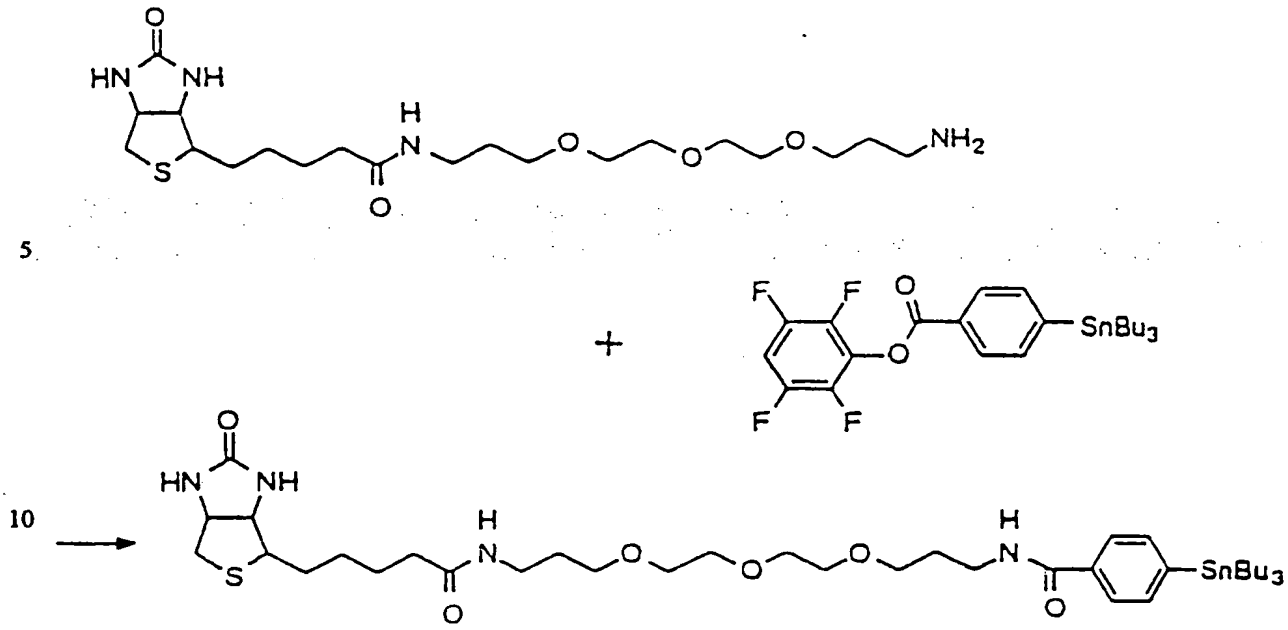


Biotin-4,7,10-trioxa-1,13-tridecanediamine (1 g, 2.24 mmol) was added to a dry flask and was dissolved in anhydrous CH_3CN/DMF (100/25 mL). To this solution was added 0.4 mL (2.9 mmol) triethylamine, followed by 0.31 g (2.7 mmol) diglycolic anhydride. The reaction was stirred at room temperature for 1 h, and the solvent was removed under vacuum. The product was triturated in 100 mL ether and was filtered. The resultant filtrate was dried under vacuum to yield 1.26 g (99%) of desired product as a colorless solid, mp 117-119°C; ^{1}H NMR ($CDCl_3$, δ): 1.3-1.7 (m, 10H), 2.1 (t, 2H), 2.7 (d, 1H), 2.8 (dd, 1H), 3.1 (m, 1H), 3.2-3.3 (m, 4H), 3.4-3.55 (m, 13H), 3.99 (s, 2H), 4.07 (s, 2H), 4.28 (m, 1H), 4.43 (m, 1H), 5.9 (s, 1H), 6.8 (m, 2H), 7.4 (t, 1H).

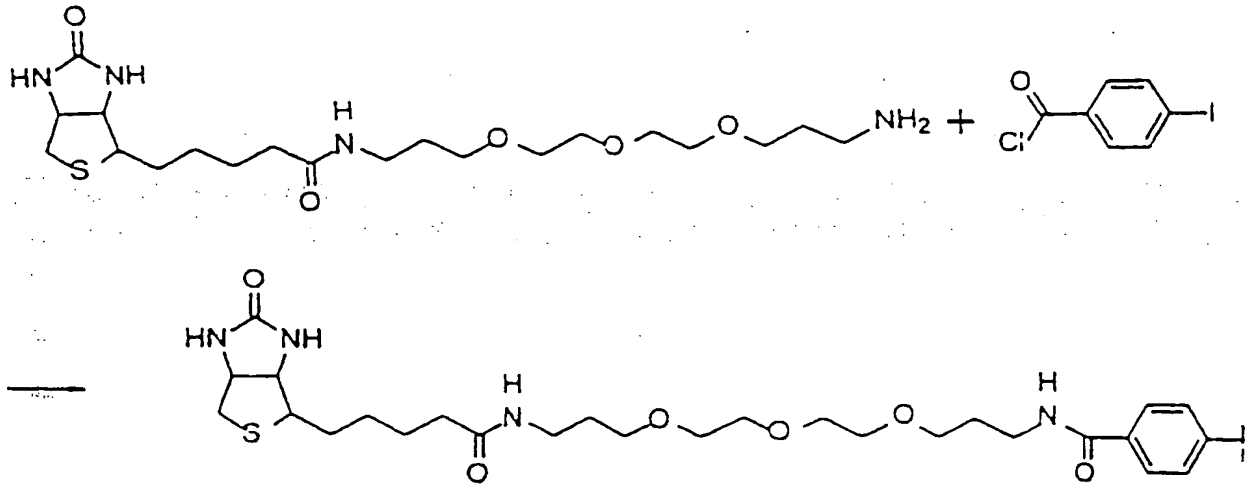
Example 5

Method for Preparation, and Radiohalogenation, of a Water Soluble Biotin Compound

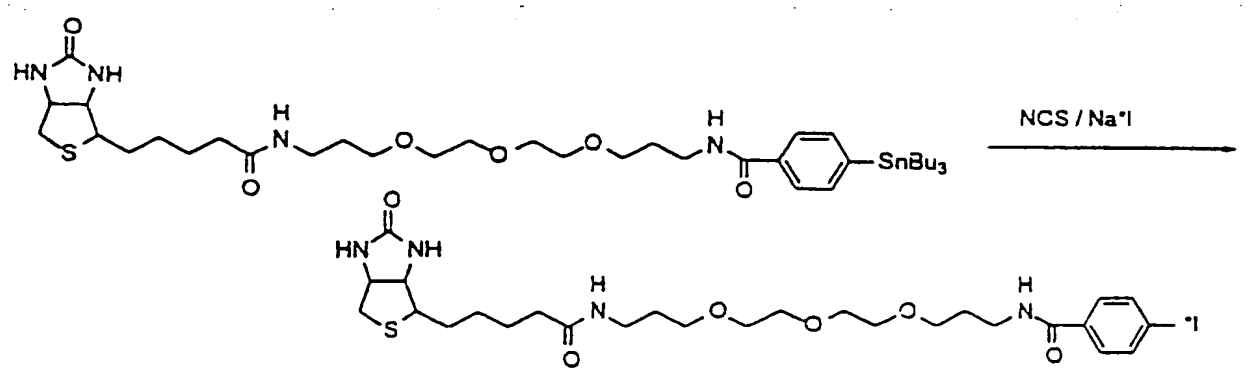
This example sets forth a general methodology for preparing a water soluble aryl stannyl biotin compound, which can be used to incorporate any number of halogens/radiohalogens into a biotin compound. The radiohalogen, iodine-125 is used, and the corresponding stable iodoaryl biotin is prepared for an HPLC standard.



Preparation of a biotin compound containing an arylstannane: TFP ester of tributyltinbenzoate (0.628 g, 1.12 mmol) was added in a dry flask and was dissolved in 10 mL of anhydrous DMF. To another dry flask containing 0.5 g (1.12 mmol) biotin-4,7,10-trioxa-1,13-tridecanediamine in 25 mL of DMF was added 0.4 mL of triethylamine. The TFP ester of tributyltinbenzoate was added dropwise to the biotin-trioxatridecanediamine solution over the period of 10-15 min. The reaction was stirred at room temperature for 2 h and solvent was removed under vacuum. The residue was extracted with 200 mL chloroform, washed with (3 x 15 mL) saturated sodium bicarbonate solution, then with (2 x 50 mL) water. The chloroform solution was dried over anhydrous sodium sulfate, then the chloroform was removed under vacuum. The resultant semisolid was dried under vacuum to yield 0.75 g (80%) of the desired product. ^{1}H NMR (MeOH, δ): 0.8 (m, 9H), 1.0-1.8 (m, 32H), 2.08 (t, 2H), 2.6 (d, 1H), 2.7 - 2.9 (m, 3H), 3.05-3.2 (m, 3H), 3.34-3.5 (m, 12H), 4.2 (m, 1H), 4.35 (m, 1H), 7.4 (d, 2H), 7.7 (d, 2H); IR (KBr, cm^{-1}): 3280, 2910, 2850, 1690, 1640, 1580, 1110.



Preparation of a biotin compound containing an aryl iodide: Biotin - 4,7,10-trioxa-1,13-tridecane-diamine (0.5 g, 1.12 mmol) was added to a dry flask and was dissolved in 25 mL anhydrous DMF. Triethylamine (0.4 mL, 2.9 mmol) was added to the solution followed by 0.266 g (1.1 mmol) *p*-iodobenzoyl chloride. The reaction was stirred overnight at room temperature, and the solvent was removed under vacuum. The residue was extracted with 200 mL chloroform and the chloroform was washed with (2 x 15 mL) water. The chloroform solution was dried over anhydrous sodium sulfate, then the chloroform was removed under vacuum. The resultant residue was dried under vacuum to yield 0.6 g (80%) of the desired product as a colorless solid, mp 107-109°C. ^{1}H NMR (MeOH, δ): 1.46 (m, 2H), 1.6-1.9 (m, 9H), 2.2 (t, 2H), 2.7 (d, 1H), 2.80 - 2.95 (m, 3H), 3.2-3.3 (m, 4H), 3.4-3.6 (m, 14H), 4.3 (m, 1H), 4.5 (m, 1H), 7.5 (d, 2H), 7.8 (d, 2H); IR (KBr, cm^{-1}): 3280, 2910, 2850, 1690, 1640, 1580, 1110; HRMS: calcd for $C_{27}H_{41}N_4O_6SI$ (M+H) is 677.1869; found 677.1865

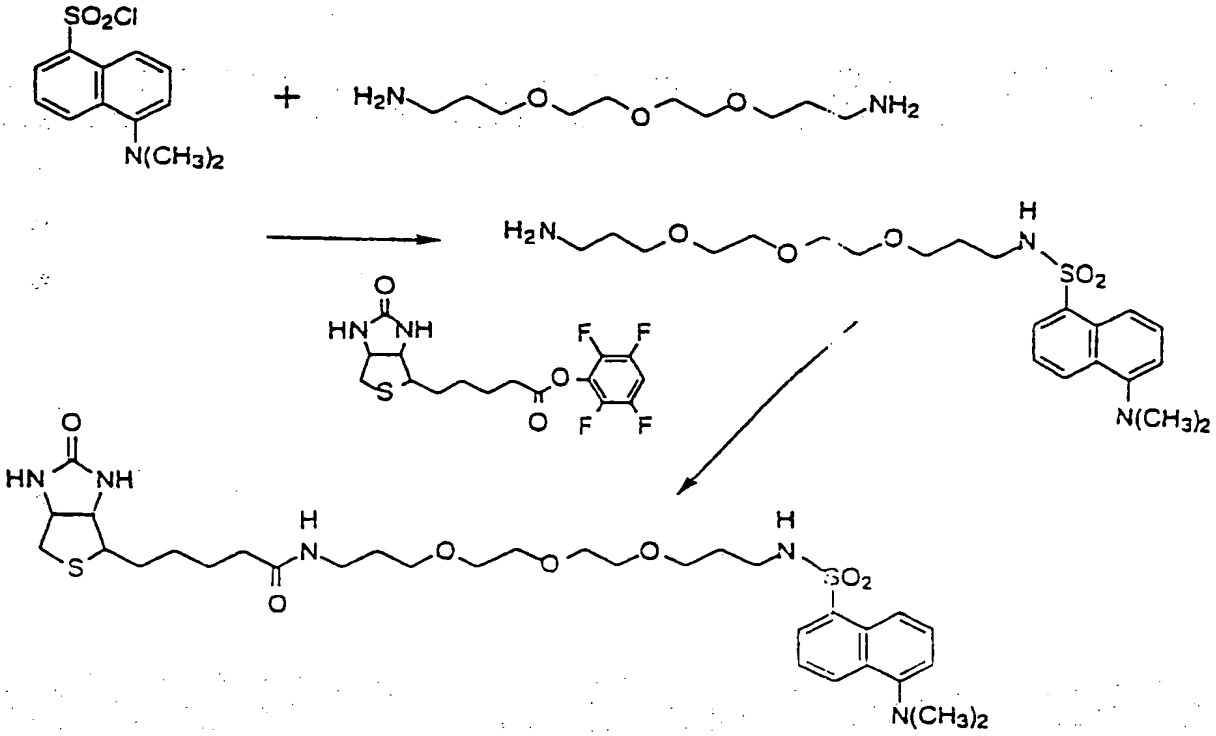


Radioiodination procedure: A 50 μL quantity of a 1 mg/mL solution of biotin-trioxadiamine-tributyltinbenzoate in MeOH/5% HOAc was placed in a small vial. To that solution was added (via syringe), 1 μgL of $Na^{125}I$ solution (275 μCi in 0.1N NaOH), followed by 10 μL of a 1 mg/mL solution of N-chlorosuccinimide in MeOH. After 5 min. at r.t., 10 μ L of sodium metabisulfite (1mg/mL in water) was added to quench the reaction. The entire reaction mixture was then injected on the HPLC and 275 μCi* was collected (peak at 4.2 min). The HPLC conditions were: an Alltima C18 column (Alltech); a gradient that began with 70% MeOH / 30% water for 2 min; increased to 100% MeOH over the next 13 minutes; then was held at 100% MeOH for 10 min. [Note: safety precautions for handling of radioiodine must be followed; *The quantity of iodine-125 measured can vary by the thickness of vessel walls that it is measured in]

The following three examples set forth general methodology for preparing a water soluble UV or fluorescence active biotin compound. Two approaches to the synthesis can be taken: 1) the linker is reacted with the UV or fluorescent molecule first, then conjugated with the biotin or modified biotin; and 2) the linker conjugate of biotin, or modified biotin, is reacted with an activated group on the UV active or fluorescent molecule. An example of each of these approaches is provided below. In the first example, the UV active dansyl molecule, activated towards reaction as the sulfonyl chloride derivative, is reacted with 4,7,10-1,13-tridecanediamine to yield the dansyl adduct, then this adduct is reacted with the TFP ester of biotin to yield a water solubilized UV active dansyl-biotin derivative, **33**.

Example 6

Method for Preparing UV Active Derivative of a Water Soluble Biotin Compound



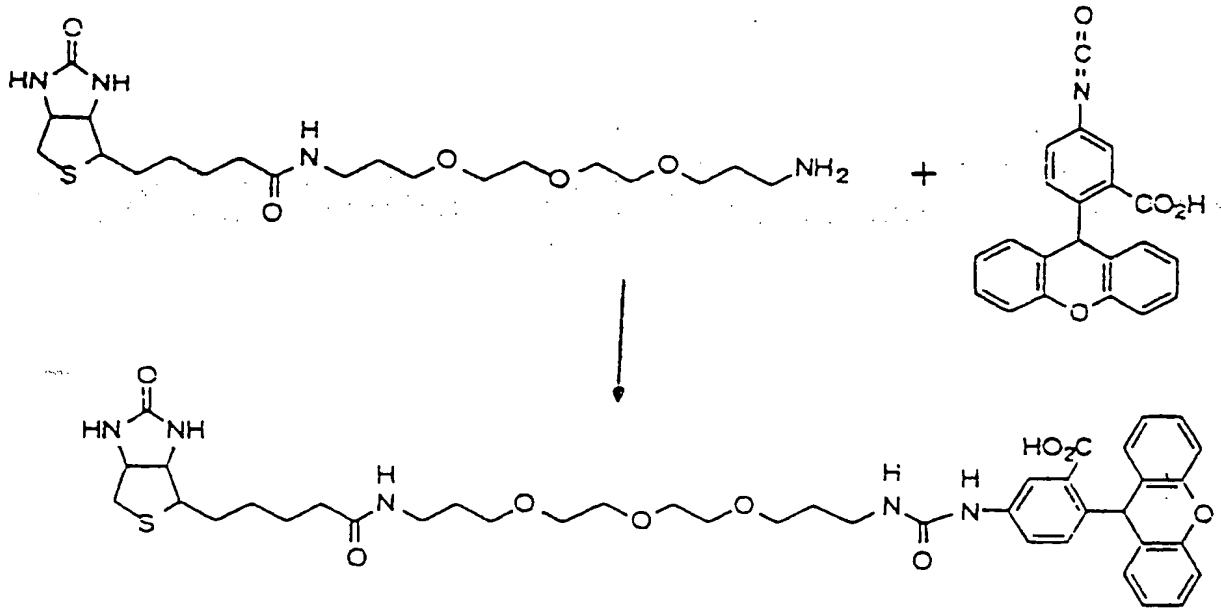
Reaction Step 1: Dansyl chloride (0.5 g, 1.85 mmol) was added in a dry flask and was dissolved in 50 mL of anhydrous ethyl acetate. In another dry flask containing 4.0 g (18.5 mmol) 4,7,10-trioxa-1,13-tridecanediamine was added 0.5 mL of triethylamine. The solution of dansyl chloride was added dropwise to the trioxatridecanediamine solution over the period of 1 h. The reaction was stirred at room temperature for 30 min and solvent was removed under vacuum. The resulting oil was triturated in 200 mL of ether, and was stirred for 30 min. The solid was filtered. The product was dissolved in ethyl acetate and loaded on silica gel chromatography column. The column was first eluted with ethyl acetate followed by ethyl acetate:methanol. Finally the product was eluted with ethyl acetate:methanol (7:3). Fractions containing product were collected, and the solvent was removed by distillation. The isolated product was dried under vacuum to yield 0.2 g (24%) of the desired product as a solid. ^{1}H NMR (MeOH, δ): 1.6 (m, 2H), 1.9 (m, 2H), 2.8-3.1 (m, 10H), 3.3 (m, 5H), 3.4-3.6 (m, 10H), 7.3 (d, 1H), 7.5-7.6 (m, 2H), 8.16 (d, 1H), 8.3 (d, 1H), 8.56 (d, 1H).

Reaction Step 2: TFP ester of biotin (0.173 g, 0.442 mmol) was added in a dry flask and was dissolved 25 mL of in anhydrous DMF. In another dry flask containing 0.2 g (0.44 mmol) of dansyl-4,7,10-trioxa-1,13-tridecanediamine adduct was added with 0.13 mL of triethylamine. The TFP ester of biotin was added dropwise to the dansyl-trioxatridecanediamine solution over the period of 10-15 min. The reaction was stirred at room temperature for 2 h (followed by HPLC) and solvent was removed under vacuum. The residue was extracted with 200 mL chloroform, washed with (3 x 15 mL) saturated sodium bicarbonate solution, then with (2 x 50 mL) water. The chloroform solution was dried over anhydrous sodium sulfate and the chloroform was removed under vacuum. The resultant residue was dried under vacuum to yield 0.3 g (98%) of the dansyl biotin derivative as a solid, mp 61-63°C. ^{1}H NMR (MeOH, δ): 1.46 (m, 2H), 1.6-1.8 (m, 8H), 2.2 (t, 2H), 2.7 (d, 1H), 2.9-3.0 (m, 12H), 3.1-3.4 (m, 8H), 3.5-3.6 (m, 7H), 4.3 (m, 1H), 4.5 (m, 1H), 7.3 (d, 1H), 7.6 (m, 2H), 8.0 (s, 1H), 8.2 (d, 1H), 8.4 (d, 1H), 8.6 (d, 1H); IR (KBr, cm^{-1}): 3280, 2910, 2800, 1690, 1450, 1140, 1090.

Example 7

Method for Preparing a Fluorescent Derivative of a Water Soluble Biotin Compound

In this example, the biotin-linker adduct **8** is reacted with commercially available fluorescein isothiocyanate.

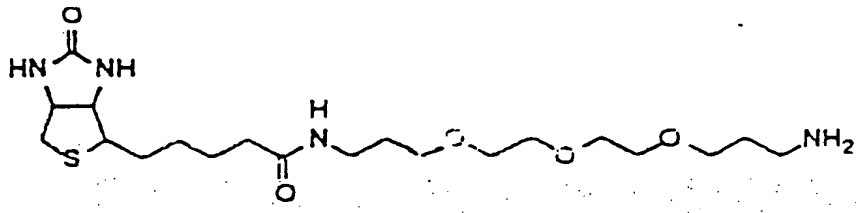


Biotin-trioxa-amine adduct **8** (0.5 g, 1.12 mmol) is dissolved in 25 mL anhydrous DMF and 0.4 mL triethylamine is added. To that solution 0.48 g (1.23 mmol) fluorescein isothiocyanate is added. The reaction mixture is stirred at room temperature for 20 h and the solvent is removed under vacuum. The residue is extracted with chloroform, and the chloroform solution is washed with water. The chloroform solution is then dried over sodium sulfate and the chloroform is removed under vacuum. The resultant solid is dried under vacuum. Purification is accomplished by silica gel column chromatography.

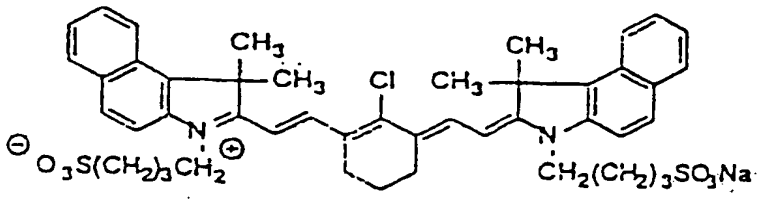
Example 8

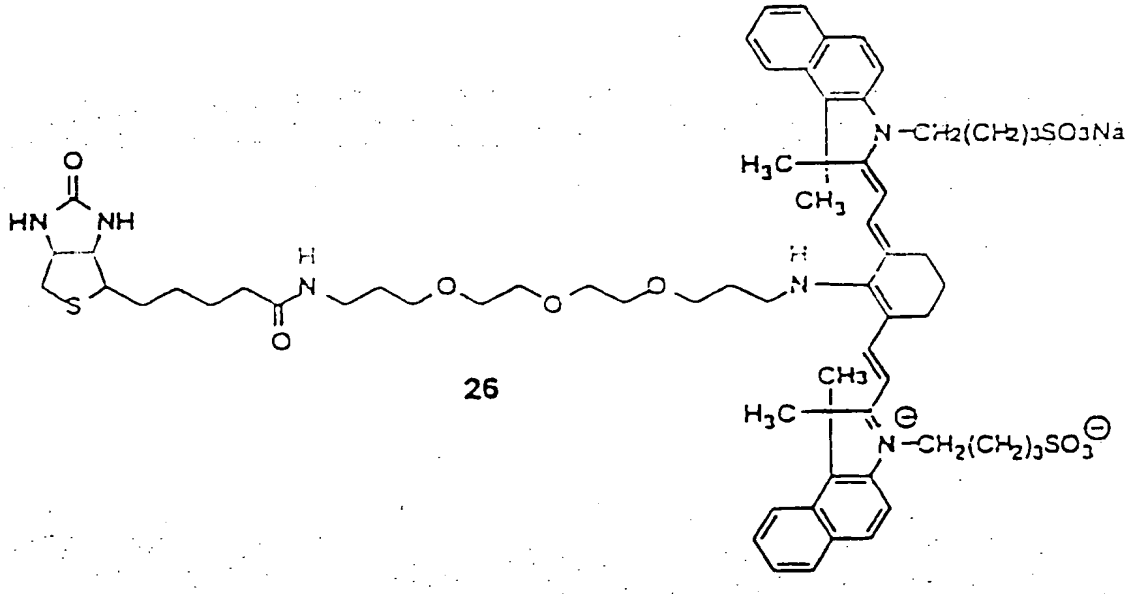
Method for Preparing a Cyanine Dye Derivative of a Water Soluble Biotin Compound

In this example, biotin-linker adduct **8** is reacted with the rigidized cyanine dye, prepared by the method of Lipowska (*Synth. Commun.* 3087-3094, 1993)



8





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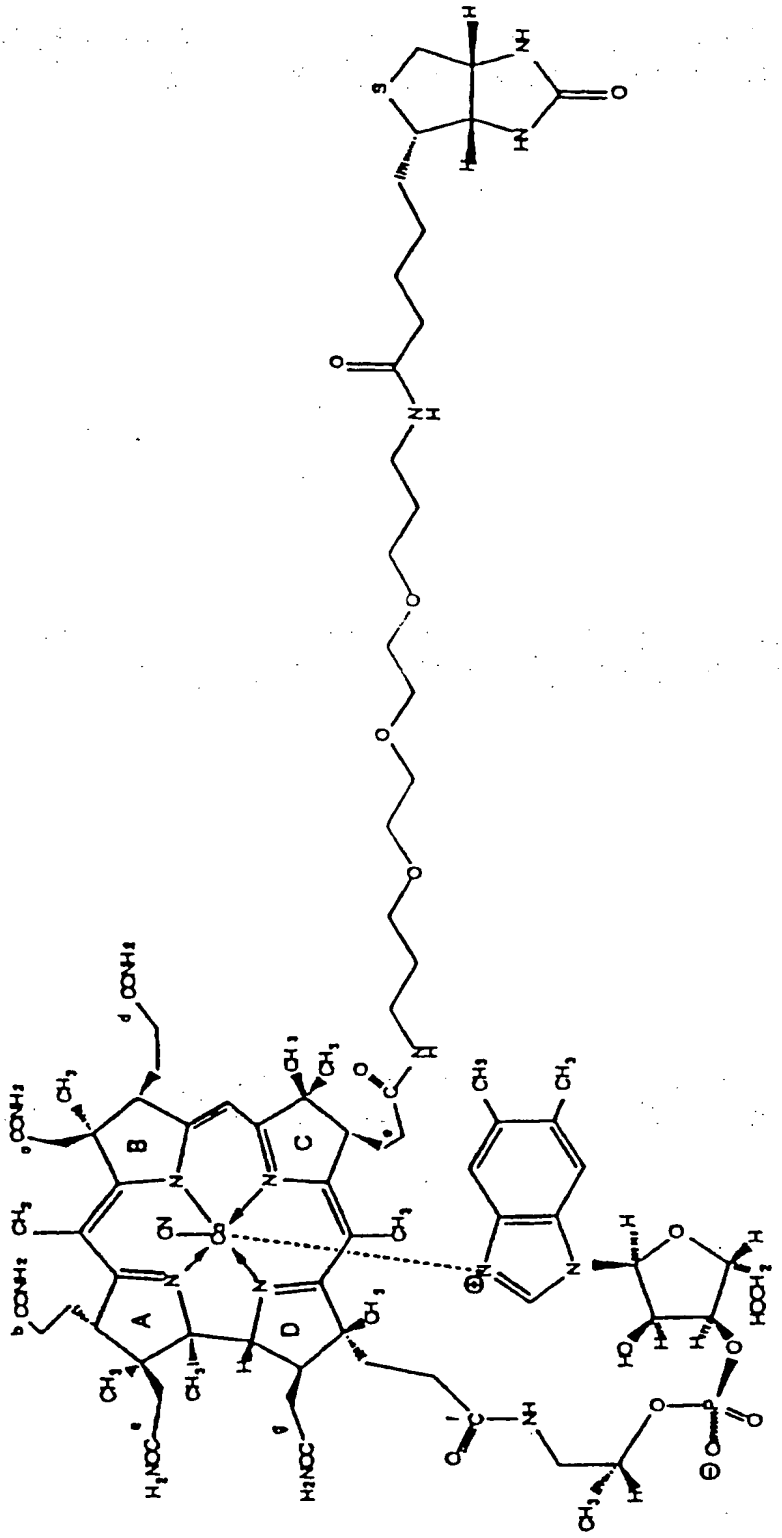
Biotin-trioxa-amine adduct **8** (0.5 g, 1.12 mmol) is dissolved in 25 mL anhydrous DMF and 0.4 mL triethylamine is added. To that solution 1.25 mmol rigidized cyanine dye is added. The reaction mixture is stirred at room temperature for 30 min and the DMF is removed under vacuum, keeping the bath temperature <50°C. The residue is triturated with ether to give the desired biotin-cyanine adduct, **26**. Purification is accomplished by column chromatography.

Example 9

Method for Preparing a Water Soluble Cobalamin-Biotin Conjugate



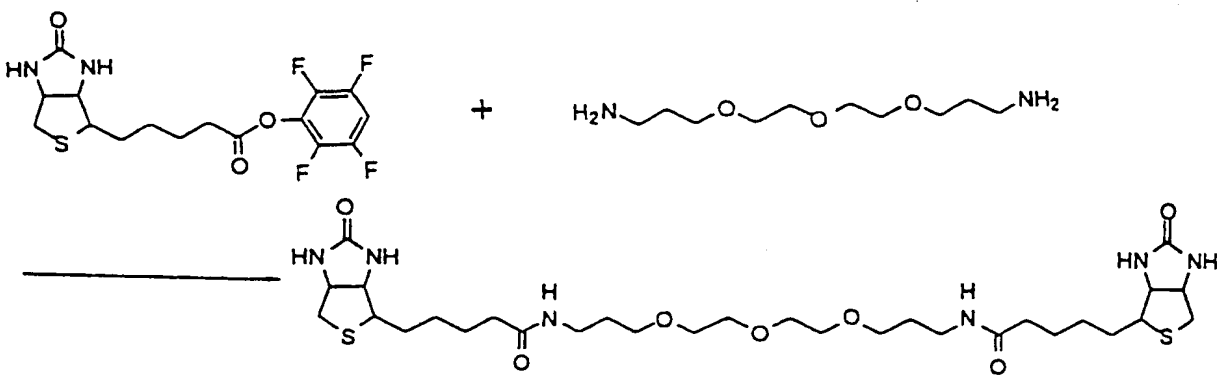
Reaction Step 1: A 2.0 g (1.47 mmol) quantity of a cyanocobalamin monocarboxylic acid, **2** or **4**, and 0.68 g (5.9 mmol) of N-hydroxysuccinimide (NCS) were dissolved in 100 mL of water. To that mixture was added 1.46 g (29 mmol) of NaCN, then 16 g (36 mmol) of 4,7,10-trioxa-1,13-tridecanediamine was added, and the pH was adjusted to 6 with 1 N HCl. To that solution was added 1.14 g (5.9 mmol) of N-ethyl-N'-dimethylaminopropylcarbodiimide hydrochloride (EDC) and the pH of the solution was readjusted to 5.5. The reaction mixture was then stirred overnight in the dark at room temperature. In 5 intervals of 6 to 14 h, 0.68 g of NHS and 1.14 g of EDC were added to the solution, readjusting the pH value to 5.5 each time. After a total reaction time of 4 days, the solution was evaporated to dryness. The residue was triturated with 100 mL of acetone and the solvent was decanted. The remaining solid was dissolved in 50 mL of H_2O and applied to an Amberlite XAD-2 (200 g; 4 cm x 60 cm) column. The column was eluted with 1 L water, then the desired product was eluted with 500 mL methanol. The methanol fractions were evaporated to dryness, and the residue was dissolved in 25 mL of water and was applied to a ion exchange column (100 g; 2.5 cm x 60 cm; acetate form; 200-400 mesh). The final product was eluted using 250 mL water, thereby leaving non-converted acid bound to the column, which was later eluted with 0.04 mol/L sodium acetate buffer pH 4.7. The fractions containing the final product were evaporated to dryness, then washed with acetone and filtered. The solid obtained was recrystallized from aqueous acetone. <u>e-isomer</u>: yield: 1.5 g (65%); mp: 112-116ûC with decomposition. ^{1}H NMR (MeOH-*d4*, δ): 0.44 (s, 3H); 1.18 (s, 3H); 1.25 (d, 5H); 1.37 (d, 8H); 1.45 (s, 4H); 1.74 (m, 10H); 1.88 (s, 11H); 2.28 (d, 7H); 2.3 (m, 15H); 2.56(d, 11H); 3.17 (t, 3H); 3.2 (t, 4H); 3.3 (m, 11H); 3.4 (m, 4H); 3.5 (s, 7H); 3.58 (d, 3H); 3.6 (m, 5H); 3.7 (m, 1H); 4.0 (m, 1H); 4.1 (d, 1H); 4.19 (m, 1H); 4.3 (m, 1H); 4.5 (d, 1H); 4.6 (m, 1H); 6.05 (d, 1H); 6.27 (s, 1H); 6.57 (s, 1H); 7.1 (d, 1H); 7.25 (s, 1H); MS (FAB$^+$): mass calcd for $C_{73}H_{109}N_{15}O_{18}CoP$ is 1557, found 1558 (M+H). IR (KBr, cm^{-1}): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060; UV (H_2O): λ361 (e = 12800);

Reaction Step 2: To a solution containing 300 mg (0.193 mmol) of cyanocobalamin-trioxadiamine in 25 mL DMF was added 30 µL triethylamine. To that solution was added 76 mg (0.212 mmol) of TFP ester of biotin. The reaction was stirred at room temperature overnight. After evaporating to dryness the solid was dissolved in 10 mL of a 1:1 methanol/H_2O mixture, and was applied to a preparative reverse phase column (25 mm x 500 mm) which was eluted with the same solvent. The fractions containing the final product were evaporated to dryness. e-acid: yield: 96 mg (35%). ^1H NMR (MeOH-d_4, δ): 0.44 (s, 3H), 1.17 (s, 3H), 1.24 (d, 4H), 1.36 (d, 8H), 1.44 (s, 3H), 1.73 (m, 5H), 1.87 (m, 3H), 2.26 (d, 6H), 2.56 (d, 9H), 3.17-3.35 (m, 10H), 3.5-3.7 (m, 12H), 3.88 (m, 1H), 4.07 (m, 1H), 4.1 (m, 1H), 4.18 (m, 1H), 4.3 (m, 2H), 4.47 (m, 1H), 4.53 (m, 1H), 4.65 (m, 1H), 6.04 (s, 1H), 6.27(d, 1H), 6.56 (s, 1H), 7.12 (s, 1H), 7.25 (s, 1H). MS (FAB+): m/e 1785 (M+1); IR (KBr, cm^{-1}): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060.

The following two examples set forth a general methodology for preparing water soluble dimers of biotin moieties. The length between the two biotin carboxylates (when fully extended) is important, and the examples therefore describe reactions containing two different linker lengths. The same reaction conditions provide dimers of different biotin moieties, and provide dimers wherein the terminus of the linker are hydroxyls or one hydroxyl and one amine. In example 10, the biotin dimer has a distance between the carboxylates which allows cross-linking (and polymerization) of biotin-binding proteins.

Example 10

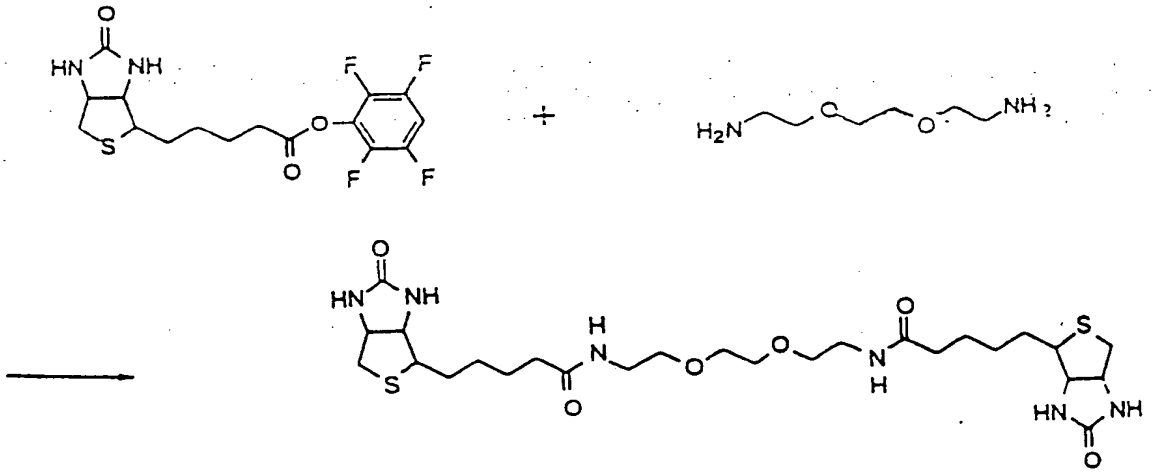
Method for Preparing a Water Soluble Biotin Dimer with a Linker of 15 Atoms



Preparation of 4,7,10-Trioxa-tridecane-1,13-N-dibiotinamide: To a solution containing 1.00 g (2.54 mmol) of biotin TFP ester in 150 mL CH_3CN at 55 °C, was added 0.28 g (1.27 mmol) trioxadiamine with stirring. The mixture was cooled to 0 °C and the precipitate formed was filtered. Crystallization from CH_3CN gave 0.65 g (76%) of the desired biotin dimer as colorless crystals. mp = 143-146 ¡C; ^1H NMR (CD_3OD, δ) 4.48 (m, 2H), 4.30 (m, 2H), 3.64 (m, 4H), 3.62 (m, 4H), 3.54 (t, 4H), 3.36 (m, 8H), 3.27 (m, 4H), 3.21 (m, 2H), 2.95 (dd, J = 12.3, 4.9 Hz, 2H), 2.72 (d, J = 12.3 Hz, 2H), 2.21 (t, 4H), 1.77 (m, 8H), 1.66 (m, 8H), 1.47 (m, 4H); MS (FAB^+): mass calcd for $C_{30}H_{52}H_6O_7S_2$ is 672; found *m/z* isotope cluster at 673-675 (M+H).

Example 11

Method for Preparing a Water Soluble Biotin Dimer with a Linker of 10 Atoms



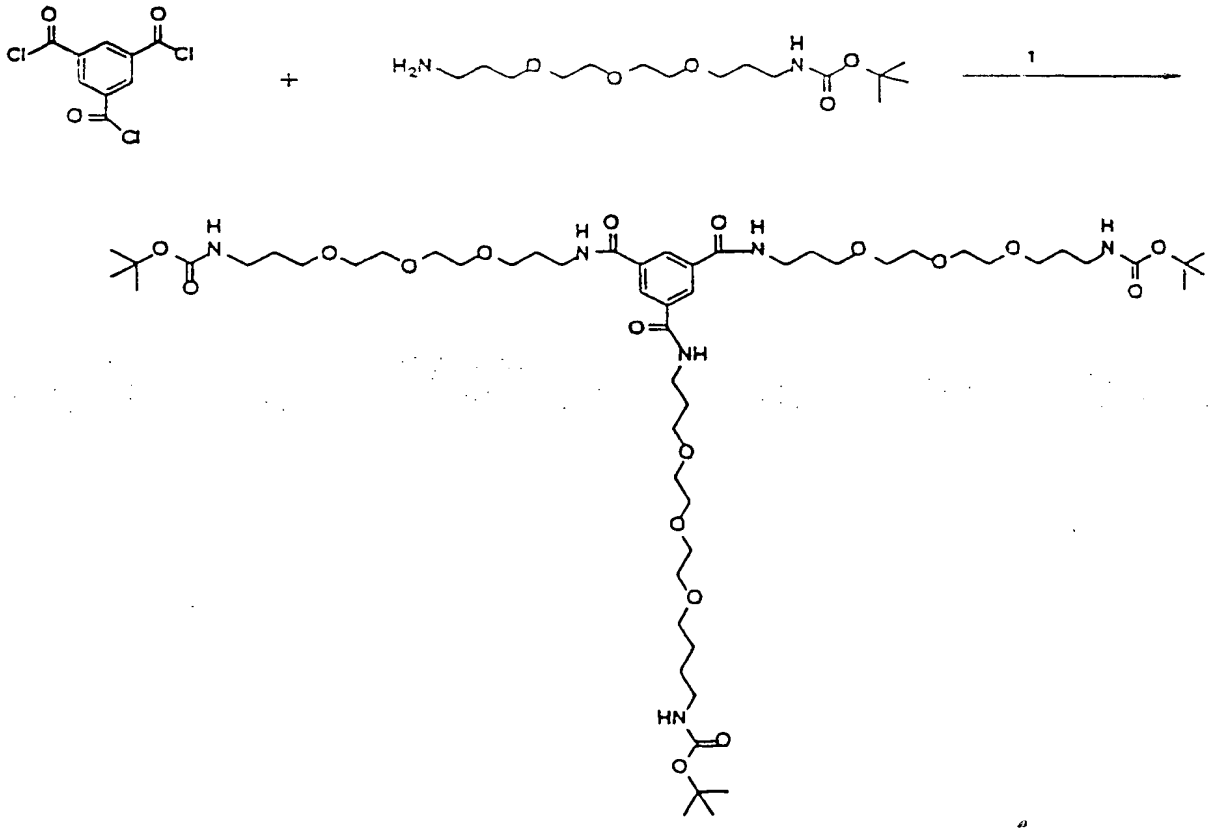
Preparation of 3,6-Dioxa-octane-1,8-N-dibiotinamide: To a solution of 1.00 g (2.54 mmol) biotin TFP ester in 150 mL CH_3CN at 55 °C, was added 0.18 g (1.27 mmol) dioxadiamine with stirring. The mixture was cooled to 0 °C and the precipitate formed was filtered. Crystallization from CH_3CN gave 0.61 g (80%) of the desired biotin dimer as colorless crystals. mp = 200-203 °C; ^1H NMR (CD_3OD, δ) 4.49 (m, 2H), 4.31 (m, 2H), 3.62 (s, NH, 4H), 3.55 (t, 4H), 3.35 (t, 4H), 3.31 (t, 4H), 2.93 (dd, J = 12.9, 4.9 Hz), 2.69

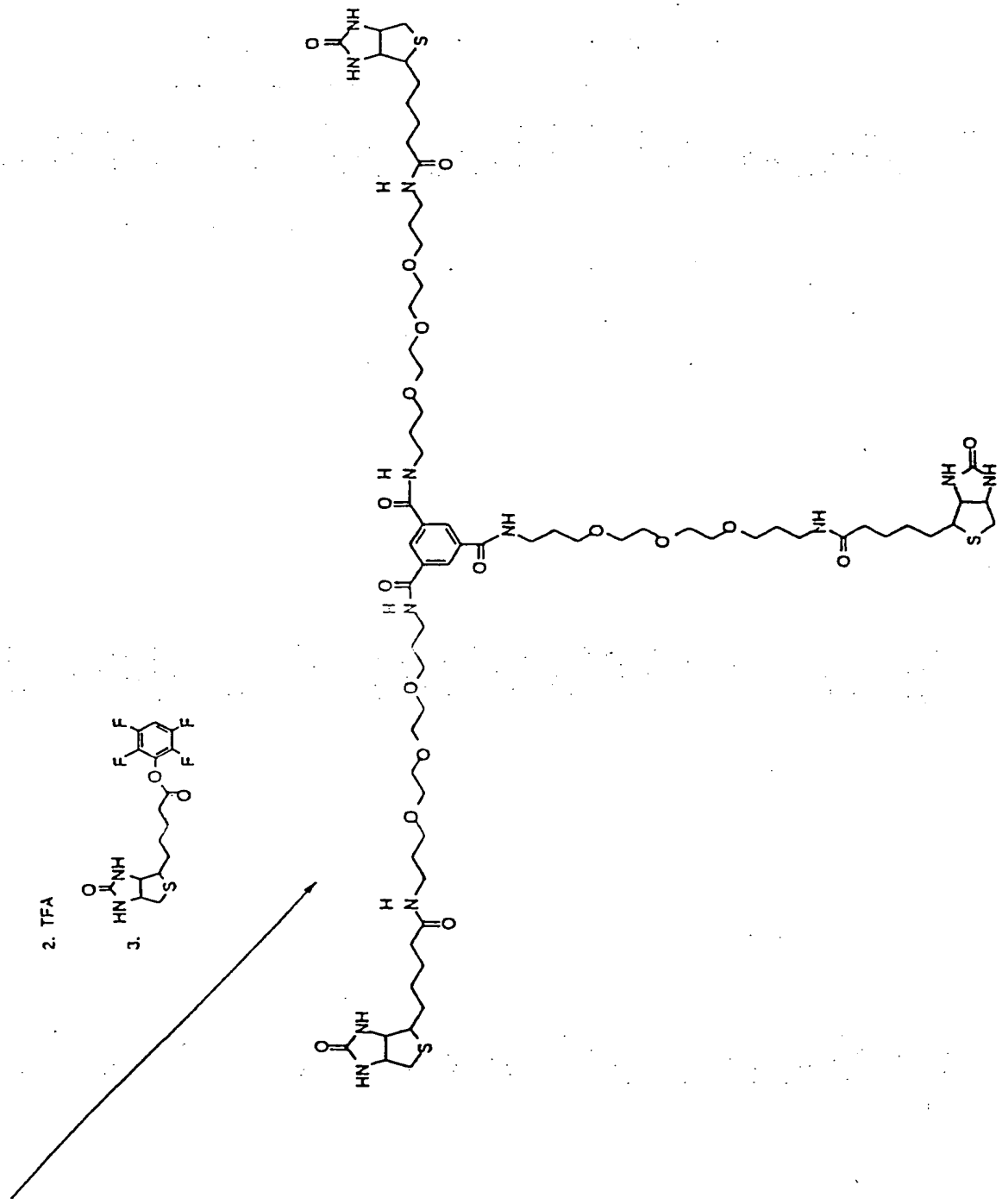
(d, J = 12.9 Hz), 2.22 (t, 4H), 1.63 (m, 8H), 1.44 (m, 4H); MS (FAB$^+$): mass calcd for $C_{26}H_{44}N_6O_6S_2$ is 600; found: *m/z* isotope cluster at 601-603 (M+H).

The following two examples set forth a general methodology for preparing water soluble trimers of biotin moieties. The length between any two of the three biotin carboxylates is important and the examples therefore describe reactions utilizing two different linker lengths. The same reaction conditions provide trimers of modified biotins, and provide biotin trimers wherein the terminus of the linker are hydroxyls or one hydroxyl and one amine. In one example the desired trimer is obtained by reaction of the water solubilizing linker with the trifunctional reagent, then addition of the biotin to that adduct. Alternatively, in the second example the biotin adduct of the water solubilizing linker (e.g. **8** or **9**) is reacted with the trimesic acid chloride to obtain the desired biotin trimer.

Example 12

Method for Preparing a Water Soluble Biotin Trimer with a 35 Atom Distance Between the Biotin Carboxylates

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3.

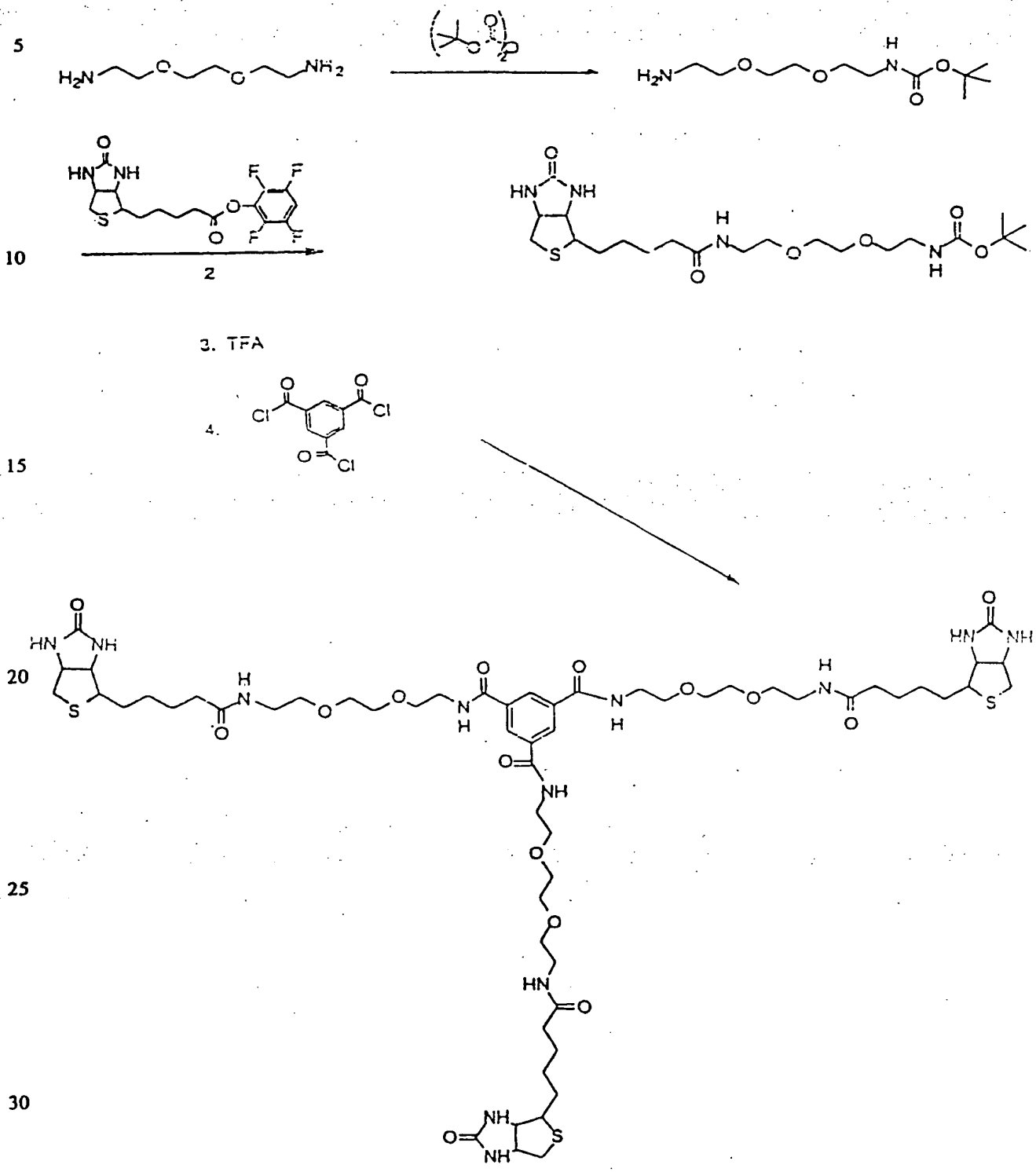
Reaction Step 1, Preparation of N,N',N"-tris-(13-N-BOC-4,7,10-trioxa-tridecanyl)benzene-1,3,5-tricarboxyamide: To a solution of benzene-1,3,5-tricarbonyl trichloride (0.28 g,1.05 mmol) in 10 mL of CH_2Cl_2 at 0 °C was added 0.96 g (2.99 mmol) of 13-N-BOC-4,7,10-trioxa-tridecaneamine [see Example 3] and 1.45 g (14.34 mmol) triethylamine in 5 mL CH_2Cl_2. That mixture was stirred under argon for 10 h at ambient temperature and the volatile materials were removed under reduced pressure. The residue was redissolved in 150 mL CH_2Cl_2, washed with 20 mL of water, dried over anhydrous Na_2SO_4 and evaporated to give 0.94 g (83%) the desired biotin trimer as a tacky solid. ^{1}H NMR ($CDCl_3$, δ) 8.38 (s, 3H), 7.64 (s, ArCONH, 3H), 5.16 (s, BOC-NH, 3H), 3.62 (m, 30H), 3.55 (m, 6H), 3.46 (m, 6H),1.89 (m, 6H), 1.67 (m, 6H), 1.41 (s, 27H).

Reaction Step 2: N,N',N"-tris-(13-amino-4,7,10-trioxa-tridecanyl)benzene-1,3,5-tricarboxamide trifluoroacetic acid salt: The N-BOC protected triamide (0.25 g, 0.23 mmol) was dissolved in 5 mL trifluoroacetic acid at ambient temperature and stirred for 30 min. Excess trifluoroacetic acid was evaporated under reduced pressure to give 0.26 g (0.22 mmol) of the trifluoroacetic acid salt as an oil: ^{1}H NMR (CD_3OD) δ 8.47 (s, 3H), 3.64 (m, 36H), 3.60 (m, 6H), 3.14 (m, 6H), 1.94 (m, 12H).

Reaction Step 3: N,N'N"-tris(13-N-Biotinamide-4,7,10-trioxa-tridecanyl)benzene-1,3,5-tricar-boxamide: To 0.19 g (0.18 mmol) of the tris trifluoroacetate salt in 3 mL DMF was added 1 mL (7.17 mmol) triethylamine, followed by 0.24 g (0.61 mmol) of biotin tetrafluorophenol ester in 2 mL DMF. The solvents were evaporated under reduced pressure and the residue was washed with 2 mL water, 2 mL of a 10% solution of $NaHCO_3$, 2 mL water, then 2 mL acetone. The residue was crystallized from methanol / acetone to give 0.04 g (11%) as colorless crystals. mp = 106-108 °C; ^{1}H NMR (CD_3OD, δ) 8.44 (s, 3H), 4.49 (m, 3H), 4.31 (m, 3H), 3.60 (m, 36H), 3.23 (m, 12H), 2.91 (m, 3H), 2.71 (d, J = 12.7 Hz), 2.15 (m, 12H), 1.91 (m, 6H), 1.65 (m, 12H), 1.40 (m, 6H); MS (FAB^+): mass calcd for $C_{69}H_{114}N_{12}O_{18}S_3$ is 1495; found: *m/z* isotope cluster at 1496-1499 (M+H).

Example 13

Method for Preparing a Water Soluble Biotin Trimer with a 25 Atom Distance Between the Biotin Carboxylates



Reaction Step 1, Preparation of 8-N-BOC-3,6-dioxa-octaneamine: To a solution of 163 g (1100 mmol) of 3,6-dioxa-1,8-octaneamine in 700 mL $CHCl_3$ was added 8.00 g (36.65 mmol) of di-tert-butyl dicarbonate in 100 mL $CHCl_3$ with stirring at ambient temperature over 30 min. The mixture was stirred for 12 h, washed with (8 x 100 mL) water, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give 8.62 g (95%) of the desired product as a colorless oil: ^{1}H NMR ($CDCl_3$, δ) 5.40 (s, 1H, CONH), 3.63 (m, 4H, -OCH_2), 3.54 (m, 4H, OCH_2), 3.32 (m, 2H, CH_2NHCO), 2.87 (t, 2H, CH_2NH_2), 1.46 (s, 9H), 1.40 (s, 1H, NH_2).

Reaction Step 2, Preparation of N-(8-N-BOC-3,6-dioxa-octanyl)biotinamide: To a solution containing 1.00 g (2.54 mmol) 2,3,5,6-tetrafluorophenol ester of biotin in 100 mL CH_3CN at 55 °C was added 0.62 g (2.50 mmol) N-BOC-4,7,10-13-trioxa-13-tridecaneamine in 20 mL CH_3CN. The mixture was allowed to cool to ambient temperature, and was stirred for 8 h. The solvent was evaporated under reduced pressure and the residue was redissolved in 150 mL EtOAc. The EtOAc solution was washed with (2 x 75 mL)10% aqueous $NaHCO_3$ and (2 x 75 mL) water, dried over anhydrous Na_2SO_4 and concentrated to give 1.03 g (87%) as a colorless solid. mp = 99-101 °C; ^{1}H NMR ($CDCl_3$) δ 6.83 (s, NH, 2H), 6.05 (s, NH, 1H), 5.21 (s, NH, 1H), 4.48 (m, 1H), 4.29 (m, 1H), 3.60 (m, 4H), 3.3.53 (m, 4H), 3.43 (m, 2H), 3.29 (m, 2H), 3.12 (m, 1H), 2.88 (dd, J = 12.8, 4.8 Hz, 1H), 2.73 (d, J = 12.8 Hz, 1H), 2.21 (t, 2H), 1.68 (m, 4H), 1.43 (s, 11H, t-Butyl and CH_2).

Reaction Step 3, Preparation of 8-N-Biotinamide-3,6-dioxa-octaneamine trifluoroacetic acid salt: The N-BOC-trioxa-biotinamide (1.03 g, 2.17 mmol) was dissolved in 10 mL trifluoroacetic acid and stirred for 30 min at ambient temperature. Excess trifluoroacetic acid was evaporated under reduced pressure to give 0.96 g (90%) of the desired product as an oil: ^{1}H NMR (CD_3OD) δ 4.10 (m, 1H), 3.90 (m, 1H), 3.28 (m, 4H), 3.24 (m, 4H)), 3.14 (m,2H), 2.95 (m, 2H), 2.79 (m, 1H), 2.51 (dd, J = 12.8, 4.8 Hz), 2.29 (d, J = 12.8 Hz), 1.81 (t, 2H), 1.25 (m, 4H), 1.02 (m, 2H).

Reaction Step 4, Preparation of N,N',N"-tris(8-N-BOC-3,6-dioxa-octanyl)benzene-1,3,5-tri-carboxamide: To a solution containing 0.28 g (1.05 mmol) of benzene-1,3,5-tricarbonyl trichloride in 10 mL CH_2Cl_2 at 0 °C was added 0.74 g (2.98 mmol) 8-N-BOC-3,6-dioxa-octaneamine and 0.70 g (6.91 nmol) triethylamine in 5 mL CH_2Cl_2. The reaction mixture was stirred under argon for 10 h at ambient temperature and the volatile material was evaporated under reduced pressure. The residue was redissolved in 150 mL CH_2Cl_2 , washed with 20 mL water, dried over anhydrous Na_2SO_4 and evaporated to give 0.82 g (87%) of the desired product as a tacky solid: ^{1}H NMR ($CDCl_3$) δ 8.33 (s, 3H), 7.67 (s, ArCONH, 3H), 5.45 (s, BOC-NH, 3H), 3.65 (m, 24H), 3.53 (m, 6H), 3.26 (m, 6H), 1.39 (s, 27H).

Example 14

Streptavidin Cross-Linking with Biotin Compounds

This experiment was designed to test streptavidin cross-linking with biotin monomer, three biotin moiety dimers comprising water soluble linkers, and th ree biotin moiety trimers comprising water soluble linkers. Each of the biotin dimers and trimers used experimentally was water soluble, that is, each biotin compound had a water solubility in excess of 0.2 mg/mL at neutral pH and ambient temperature. Biotin dimers illustrated herein as compounds **39** and **40** and trimers illustrated herein as compounds **48** and **49** were used in this experiment.

A Reacti-Bind Streptavidin Coated Polystyrene Strip Plate (Pierce) was washed with 100 mM PBS prior to use. To lane one (12-wells) was added 100 μL PBS. To lanes 2-7 were added 100 pmole of biotin dimer or biotin trimer to be tested in 100 μL PBS. Lane 8 was treated in the same manner with 100 pmoles of biotin monomer. The plate was then incubated at 37 °C with shaking for 10 minutes. All the wells were then emptied and rinsed with 100 μL PBS. The PBS was removed and 25 pmoles of ^{125}I labeled streptavidin (specific activity of 1 μCi/*ug*) was added to each well in 100 μL PBS. The plate was then incubated with shaking for another 10 minutes. The streptavidin was

removed and all the wells were washed with 100 μL PBS. After removal of the PBS, the first 3 wells of each lane were again filled with 100 μL PBS. The remaining wells were then filled with 100 μL of their respective biotin dimer or biotin trimer as in the first step. The plate was then incubated again for 10 minutes, and all wells were rinsed with PBS as before. Another 100 μL of the radiolabeled streptavidin was then added to all of the wells, 100 μL PBS was added to the first 6 wells of each lane. The remaining 6 wells in each lane were filled with 100 μL of their respective dimer or timer and again incubated for 10 minute at 37 °C . After removing the liquid in all the wells and washing with PBS, 100 μL of the radiolabeled streptavidin was again added to each well followed by incubation. The streptavidin was removed and the wells washed with PBS. The first 9 wells in each lane were then filled with 100 uL PBS and the remaining 3 wells in each lane were filled with 100 μL of their respective dimer or trimer. After incubating and washing, 100 μL of radiolabeled streptavidin was again added to each well. After incubating for 10 minutes, each well was washed with 100 μL of PBS and all liquid removed. The wells were then separated and placed in a gamma counter. The amount of activity remaining in each well was determined as a percentage of the highest initial streptavidin binding.

The Figure illustrates the percentage streptavidin binding of the biotin monomer and the dimers and trimers as a function of sequential biotin compound additions. Each of the biotin trimers used demonstrated increased streptavidin binding with each sequential biotin trimer addition. Neither the biotin monomer nor any of the biotin dimers tested demonstrated appreciable increases in streptavidin binding with sequential biotin compound additions.

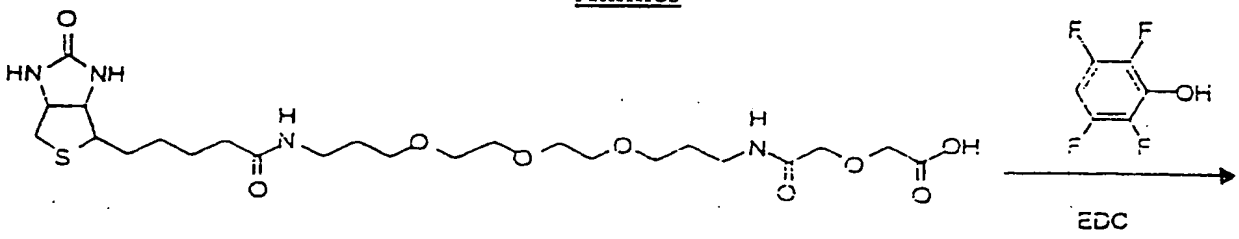
This experimental data demonstrates that biotin trimers of the present invention can be successfully used to provide amplification of binding sites for complementary binding moieties, such as streptavidin. The water solubility of such biotin dimers, trimers and multimers is important in *in vivo* applications for such compounds, such as amplification of binding sites at a preselected site, e.g. a tumor, by repeated administration of a biotin dimer, trimer, multimer compound, or combinations of such compounds.

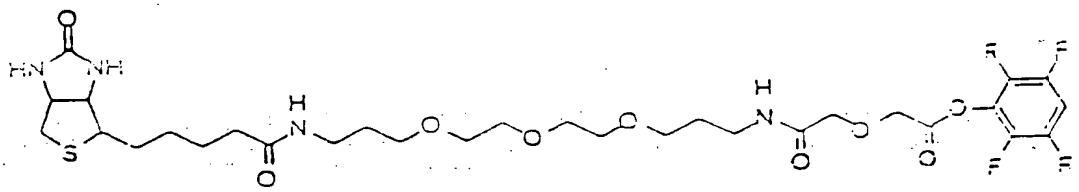
The following three examples set forth a general methodology for preparing water soluble biotin compounds which are used to biotinylate other molecules. In the first

example, the TFP ester, 24, is prepared by esterification of the biotin-trioxa-amido-glycolate, 13, with tetrafluorophenol. Under the same reaction conditions any number of activated esters can be prepared by substitution of different phenols, or other alcohols (e.g. N-hydroxysuccinimide).

Example 15

Method for Preparing a Water Soluble Biotin Compound that is Reactive with Amines

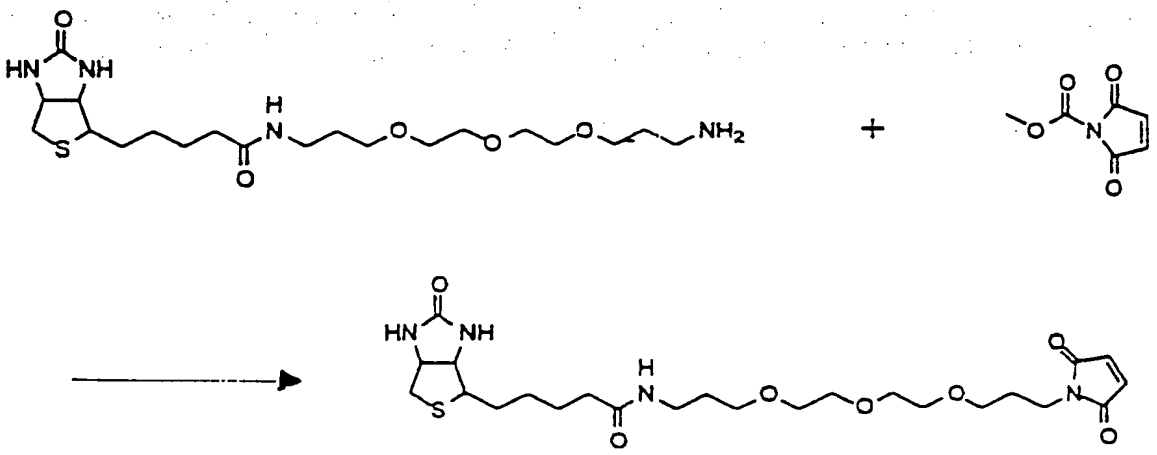




Biotin - 4,7,10-trioxa-1,13-tridecanediamine - diglycolic carboxylate (1.0 g, 1.78 mmol) was added in a dry flask and was dissolved in anhydrous CH_3CN/DMF (75/25 mL). Tetrafluorophenol (2.13 mmol, 0.353 g) was added to the solution followed by 0.41 g (2.13 mmol) EDC. The reaction was stirred at room temperature for 1 h (followed by HPLC), and the solvents were removed under vacuum. The residue was extracted with 200 mL chloroform, washed with (3 x 25 mL) saturated sodium bicarbonate solution, then with (2 x 50 mL) water. The chloroform solution was dried over anhydrous sodium sulfate and the chloroform was removed under vacuum. The product was tritrated in 100 mL ether and was filtered. The filtrate was dried under vacuum to yield 0.8 g (63%) of desired product as a solid. ^{1}H NMR ($CDCl_3$, δ): 1.3-1.8 (m, 10H), 2.1 (t, 2H), 2.7 (d, 1H), 2.8 (dd, 1H), 3.1 (m, 1H), 3.2-3.6 (m, 15H), 3.8 (t, 2H), 4.08 (s, 1H), 4.27 (m, 3H), 4.4 (m, 2H), 5.7 (s, 1H), 6.4 (s, 1H), 6.5 (m, 1H), 7.0 (m, 1H).

Example 16

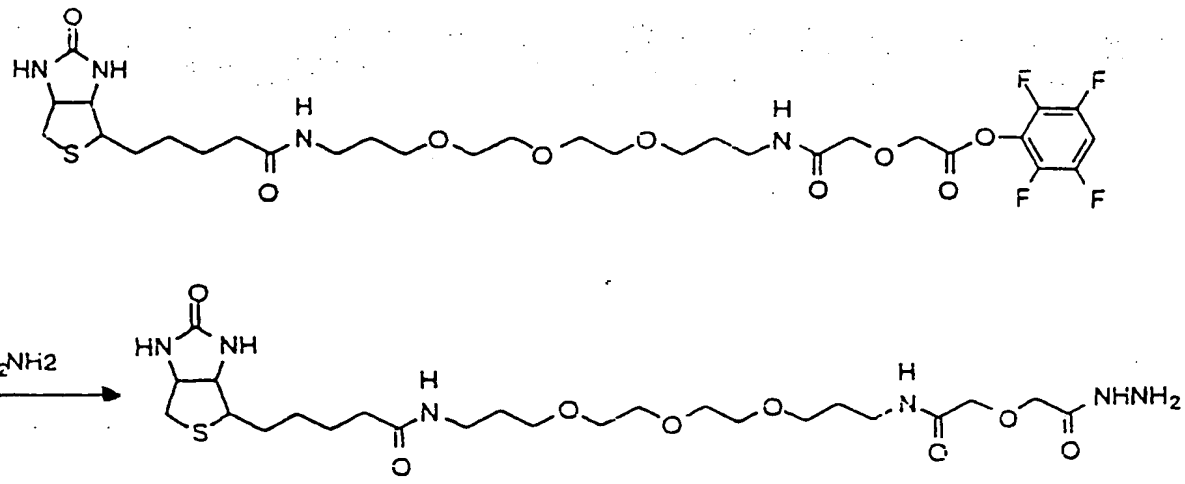
Method for Preparing a Water Soluble Biotin Compound that is Reactive with Sulfhydryls or Amines



Biotin-4,7,10-trioxa-1,13-tridecanediamine (1.0 g, 2.2 mmol) was dissolved in 12 mL of saturated aqueous sodium bicarbonate and was cooled with ice-water. N-Methoxycarbonylmaleimide (4.5 mmol, 0.696 g) was added and the reaction was stirred at 0°C for 10 min. A 50 mL quantity of water was added to the reaction and the stirring was continued at room temperature for an additional 15 min. The solution was extracted with (4 x 100 mL) chloroform. The combined chloroform extracts were washed with (2 x 50 mL) water, dried over anhydrous sodium sulfate, and chloroform was removed under vacuum. The product was triturated in 100 mL ether and was filtered. The isolated product was dried under vacuum to yield 0.57 g (49%) of the desired compound as a colorless solid; mp 112-114°C; ^{1}H NMR (MeOH, δ): 1.46 (m, 2H), 1.6-1.8 (m, 9H), 2.2 (t, 2H), 2.7 (d, 1H), 2.9 (dd, 1H), 3.2-3.3 (m, 4H), 3.5-3.6 (m, 15H), 4.3 (m, 1H), 4.5 (m, 1H), 6.8 (s, 2H); IR (KBr, cm^{-1}): 3280, 2910, 2850, 1760, 1690, 1640, 1110, 940; HRMS: calcd for $C_{24}H_{38}N_4O_7S$ (M+H) is 527.2539, found 527.2526.

Example 17

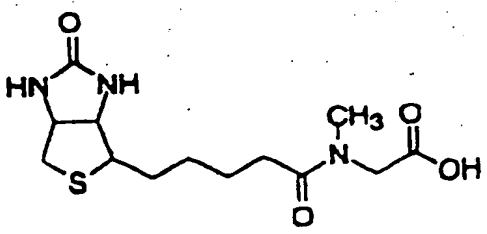
Method for Preparing a Water Soluble Biotin Compound that is Reactive with Aldehydes, Ketones, and Oxidized Carbohydrates



A solution of 0.5 g of biotin-trioxamido-glycolate TFP ester in 50 mL anhydrous THF is added dropwise to a solution containing 5 mL anhydrous hydrazine in THF at 0 °C. After the addition is complete, the reaction solution is allowed to come to room temperature over a 1 h period. The THF and excess hydrazine are removed under vacuum. The residue is triturated with 100 mL ether and the solid product is collected by filtration

Example 18

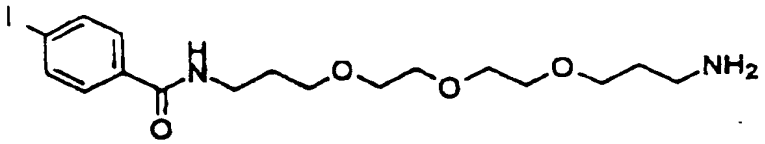
Method for Preparing a Water Soluble Biotin Compound which is Stabilized from Biotindase Cleavage and has a Radioiodine Reporter Moiety Attached



N-Methylglycylbiotinamide. A 2.5 g (6.4) mmol) quantity of biotin tetraflourophenyl ester dissolved in 30 mL DMF under argon atmosphere was added to a

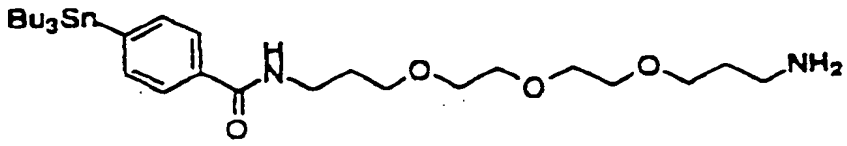
mixture of (1.075 g, 7.68 mmol) N-methylglycine methyl ester hydrochloride dissolved in 10 mL of DMF and 1.25 mL (9.0 mmol) Et_3N. The reaction mixture was stirred at rt for 2-3 h, then the solvent was removed under vacuum. The residue was extracted into CH_3Cl (2 x 100 mL). The CH_3Cl extract was washed with H_2O (2x25 mL), dried over anhydrous Na_2SO_4. The CH_3Cl was removed under vacuum and the residue was dried under high vacuum to yield 2.1 g (99.7%) of methyl ester of N-methylglycine biotinamide as a semisolid. ^1H NMR (CD_3OD) δ 4.5 (m, 1H), 4.3 (m, 1H), 3.7 (d, J = 9.6 Hz, 2H), 3.1-3.2 (m, 3H), 2.8-3.0 (m, 6H), 2.7 (d, J = 12.6 Hz, 1H), 2.2-2.5 (m, 2H), 1.4-1.8 (m, 6H).

N-Methylglyclbiotinamide methyl ester was hydrolized in a mixture of 31.5 mL MeOH and 10.5 mL of 1 N NaOH solution at rt for 1 h. Methanol was removed by distillation and the aqueous solution remainting was sitrred with ether (100 mL). This resulted in precipitation of a solid. The solid was filtered and dried under vacuum to yield 1.2 g (60%) of desired product as a colorless solid, mp 190-192 °C, ^1H NMR (DMSO-d_6) δ 6.2 (d, J = 15.6 Hz, 2H), 3.7-4.2 (m, 4H), 2.7-2.9 (m, 3H), 2.6 (m, 2H, 2.35 (m, 2H), 2.0 (m, 2H), 1.0-1.5 (m, 6H); IR (KBr, cm^{-1}) 3320, 3280, 2910, 1695, 1650, 1480, 1120, 880; HRMS: mass calcd for $C_{13}H_{22}N_3O_4S$ (M+H) is 316.1331; Found: 316.1321.

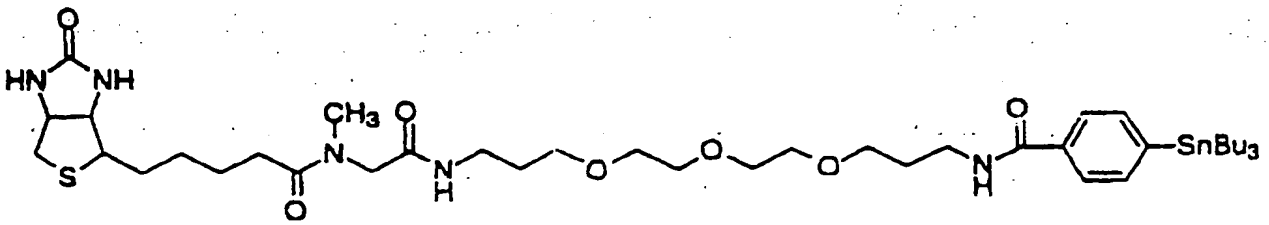


N-(13'-Amino-4',7',10' -trioxatridecanyl)-4-iodobenzamide. A 2.0 g (5.05 mmol) quantity of 3 dissolved in 100 mL anhydrous DMF was added dropwise over 1 h to a mixture of 11 g (50 mmol) 4,7,10 -trioxa-1,13-tridecanediamine and 2 mL Et_3N. The reaction was stirred at rt for 30 min and solvent was removed under vacuum with rotary evaporation. The resulting oil was triturated in 200 mL of ether. The produce was extracted with CH_3Cl (2 x 100 mL). The combined CH_3Cl extracts were washed with H_2O (2 x 50 mL), dried over anhydrous Na_2SO_4, and CH_3Cl was removed under vacuum.

The semisolid was then loaded onto a silica column (2.5 cm x 35 cm), eluting with EtOAc, then EtOAc/MeOH. The product was eluted with EtOAc:MeOH (7:3). The solvent was removed under vacuum to yield 1 g (44%) of desired product as a light yellow solid, mp 117-119°C; ^{1}H NMR ($CDCl_3$) δ 7.65 (d, J = 8.4 Hz, 2H), 7.4 (d, J = 8.8 Hz, 3H), 6.4 (s, 2H), 3.4-3.6 (m, 14H), 2.9 (t, J = 6.3 Hz, 2H), 1.6-1.8 (m, 4H); IR (KBr, cm^{-1}) 3340, 1630, 1585, 1530, 1460, 1110; HRMS: mass calcd for $C_{17}H_{28}IN_2O_4$ $(M+H)^+$ is 451.1096: Found 451.1083.

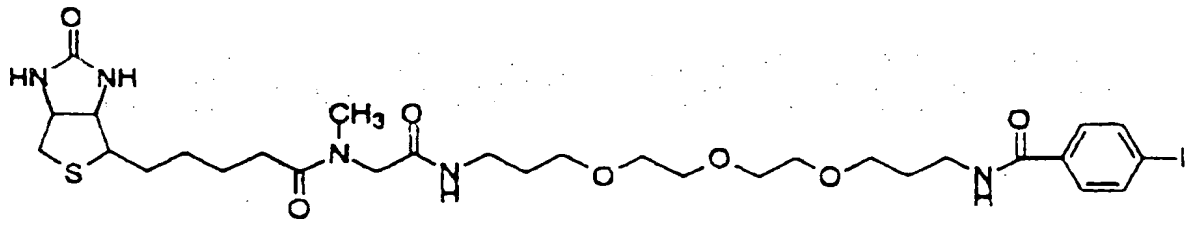


N-(13'-Amino-4',7',10'-trioxatridecanyl)-4-tributylstannylbenzamide. A 2.0 g quantity (3.57 mmol) of 4 dissolved in 50 mL anhydrous DMF was added dropwise over 1 h to a mixture containing 8 g (35.7 mmol) 4,7,10-trioxa-1,13-tridecanediamine and 1 mL Et_3N. The reaction mixture was stirred at rt for 30 min and solvent was removed under vacuum. The resulting oil was triturated in 200 mL of ether. The product was extracted with CH_3Cl (2 x 100 mL). The combind CH_3Cl extracts were washed with (2 x 50 mL) H_2O, dried over anhydrous Na_2SO4, and the CH_3Cl was removed under vacuum. The resultant oil was dried under vacuum to yield 2.2 g (99%) of desired product; ^{1}H NMR ($CDCl_3$) δ 7.7 (d, J = 7.8 Hz, 2H), 7.5 (d, J = 8.0 Hz, 3H), 3.4-3.6 (m 14H), 2.7 (t, J = 6.7 Hz, 2H), 0.6-1.8 (m, 33H); IR (KBr.cm^{-1}) 3340, 1630, 1585, 1530, 1460, 1110; HRMS mass calcd for $C_{29}H_{53}N_2O_4Sn(120)$ $(M+H)^+$ is 615.3184: Found: 615.3159.



N-(13-(*p*-tri-*n*-butylstannylbenzamido)-4,7,10-trioxatridecanyl)-N-methylglycylbiotin-amide. To a 0.25 g (0.8 mmol) quantity of N-

methylglycylbiotinamide dissolved in 15 mL DMF under argon atmosphere was added 0.25 g (p.96 mmol) 2,3,5,6-tetraflourophenyl triflouroacetate followed by 0.119 mL (0.96 mmol) Et_3N. The reaction mixture was stirred at rt for 20 min. To the solution was added 0.491 g (0.8 mmol) N-(13'-amino-4',7',10'-trioxatridecanyl)-4-tributylstannylbenzamide in 5 mL DMF. That mixture was stirred at rt for 0.5 h and the solvent was removed under vacuum. The residue was tirturated with ether and the solid was filtered. The isolated solid was dried under high vacuum to yield 0.5 g (69%) of produce as colorless solid; mp 89-91°C, ^1H NMR (CD_3OD) δ 7.7 (d, J = 7.4 Hz, 2H), 7.5 (d, J = 7.4 Hz, 2H), 4.5 (m, 1H), 4.3 (m, 1H), 4.0 (d, J = 8.4 Hz, 2H), 2.9-3.8 (m, 25H), 2.7 (d, J = 12.6 Hz, 2H), 2.4 (m, 2H), 0.8-2.0 (m, 36H); IR (KBr, cm^{-1}) 3280, 2920, 2860, 1695, 1650, 1450, 1120, 1000; HRMS: mass calcd for $C_{42}H_{74}N_5O_7SSn(120)$ $(M+H)^+$ is 912.4308; Found: 912.4331.



N'-(13-(*p*-iodobenzamido)-4,7,10-trioxatridecanyl)-N-methylglycylbiotinamide. To a 0.15 g (0.48 mmol) quantity of N-methylglycylbiotinamide dissolved in 12 mL DMF under argon atmosphere was added 0.15 g (0.576 mmol) 2,3,5,6-tetraflourophenyl triflouroacetate and 71μL (0.576 mmol) Et_3N. The reaction mixture was stirred at rt for 20 min. To the solution was added 0.216 g (0.48 mmol) N-(13'-amino-4',7',10'-trioxatridecanyl)-4-iodobenzamide in 5 mL DMF. The reaction mixture was stirred at rt for 0.5 h. then the solvent was removed by distillation, and the residue was loaded onto a silica gel column. The column was eluted with EtOAc, then with increasing amounts of MeOH in EtOAc. The fractions containing the product were evaporated to dryness to yield 0.22 g (61 %) of 24 as an colorless solid

after drying under high vacuum: mp 114-4.3 (m,1H), 4.0 (d, J = 10 Hz, 2H), 2.9-3.6 (m, 22H), 2.9 (m, 2H), 2.7 (d, J = 12.6 Hz, 1H), 2.4 (m, 2H), 1.3-1.9 (m, 11H); IR (KBr, cm^{-1}) 3280, 2920, 2860, 1695, 1650, 1450, 1120, 1000; HRMS: mass calcd for $C_{30}H_{46}IN_5O_7SNa$ $(M+Na)^+$ is 770,2060; Found: 770.2086.

Procedure for Radioiodination of N'-(13-(*p*-tri-*n*-butylstannylbenzamido)-4,7,10-trioxatridecanyl)-N-methylglycylbiotinamide to prepare N'-(13-(*p*-[^{125}I]iodobenzamido)-4,7,10-trioxatridecanyl)-N-methylglycylbiotinamide. To 50 μL of a 1 mg/mL solution of stannylbenzamidobiotin derivative was added 1-5 μL of Na[^{125}I]I in 0.1 N NaOH, followed by 10 μL of a 1 mg/mL solution of NCS in MeOH. After 2 min, 10 μL of a 1 mg/mL aqueous sodium metabisulfite solution was added. The reaction mixture was then drawn into a syringe for purification by HPLC. The radioiodinated compounds were obtained from the HPLC effluent. Radiochemical yields of the isolated (purified) biotin derivatives were 37%-39%.

We Claim:

1. A biotin-containing compound comprising a biotin moiety joined to a water soluble linker moiety.

2. A biotin-containing compound according to claim1, wherein the water soluble linker moiety is non-ionized.

3. A biotin-containing compound according to claim 2, wherein the non-ionized water soluble linker moiety comprises one or more moiety selected from the group consisting of: ethers, hydroxyls, aminos, thioethers, and thiols.

4. A biotin-containing compounds according to claim 3, wherein the non-ionized water soluble linker moiety comprises one or more moiety selected from the group consisting of: polyhydroxy, polyamino, polyether, polyphosphoric acid, polyalcohol and polyamine moieties.

5. A biotin-containing compound according to claim 1, wherein the water soluble linker is ionized or ionizable.

6. A biotin-containing compound according to claim 5, wherein the ionized or ionizable water soluble linker moiety comprises one ore more moieties selected from the group consisting of: anionic boron cage moieties, aryl sulfonates, and aryl amonium ions.

7. A biotin-containing compound according to claim 1, wherein the water soluble linker moiety is about 8 to 20 atoms in length.

8. A biotin-containing compound according to claim 1, wherein the water soluble linker moiety is a branched-chain moiety.

9. A biotin-containing compound according to claim 1, additionally comprising a reactive moiety.

10. A biotin-containing compound according to claim 9, wherein the reactive moiety is selected from the group consisting of: carboxylate esters, amino reactive moieties, imidate esters, isocyanates, isothiocyanates, alpha-haloacetamides, aldehydes, sulfhydryl reactive moieties, oxidized carbohydrate reactive moieties, and pH- photo or heat-activated reactive moieties.

11. A biotin-containing compound according to claim 1, additionally comprising a cleavable functional moiety.

12. A biotin-containing compound according to claim 1, additionally comprising a stabilizing moiety that inhibits cleavage of the compound by biotinidase.

13. A biotin-containing compound according to claim 1, additionally comprising a cross-linking moiety.

14. A biotin-containing compound according to claim 1, additionally comprising a targeting moiety, joined to the linker moiety.

15. A biotin-containing compound according to claim 1, additionally comprising an imageable moiety joined to the linker moiety.

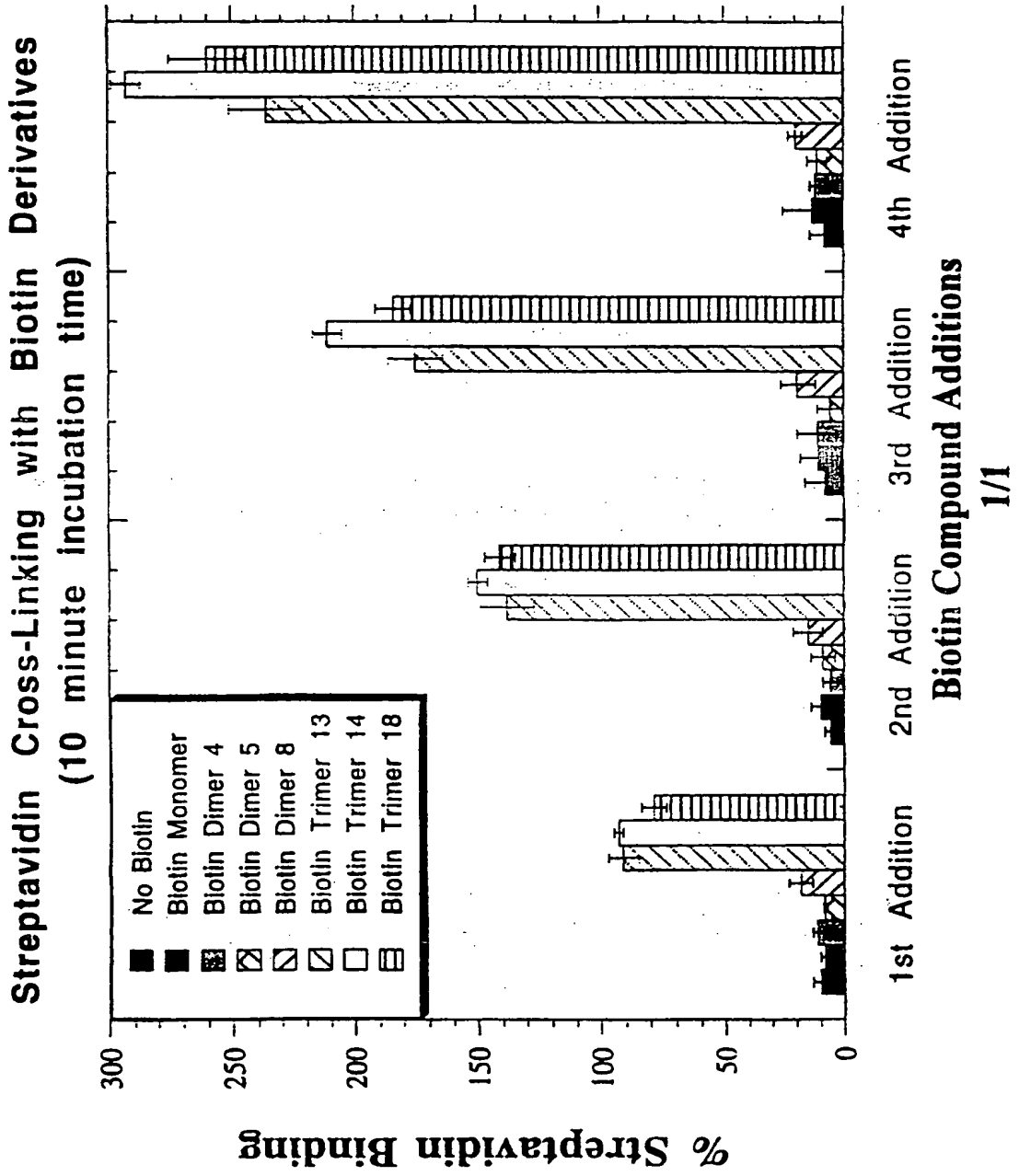
16. A biotin-containing compound according to claim 1, additionally comprising a diagnostic moiety joined to the linker moiety.

17. A biotin-containing compound according to claim 1, additionally comprising a therapeutic moiety joined to the linker moiety.

18. A biotin-containing compound according to claim 1, additionally comprising a radionuclide moiety.

19. A biotin-containing compound comprising at least two biotin moieties and at least one non-aliphatic water soluble linker moiety.

20. A biotin-containing compound according to claim 1, comprising three or more biotin moieties.


Streptavidin Cross-Linking with Biotin Derivatives
(10 minute incubation time)
No Biotin
Biotin Monomer
Biotin Dimer 4
Biotin Dimer 5
Biotin Dimer 8
Biotin Trimer 13
Biotin Trimer 14
Biotin Trimer 18
% Streptavidin Binding
300
250
200
150
100
50
0
1st Addition
2nd Addition
3rd Addition
4th Addition
Biotin Compound Additions

1/1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/02560

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 15/00; C12P 13/18; C07D 235/02; A01N 43/52; A61K 51/00
US CL :435/11; 424/9.4; 514/387; 548/304.1; 536/17.4
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/11; 424/9.4; 514/387; 548/304.1; 536/17.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, REGISTRY, WPIDS, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DRESSENDORFER et al. A Non-Isotopic Immunoassay for Guanosine 3':5'-Cyclic Monophosphate Using a Cyclic GMP-Biotin Conjugate as Tracer. Journal of Immunoassay. 1995, Vol.16, No.1, pages 37-53.	1-16
X ---- Y	LEVENSON et al. Biotinylated Psoralen Derivative for Labeling Nucleic Acid Hybridization Probes. Methods in Enzymology. 1990, Vol.184, pages 577-587.	1-15 ------- 19-20
X	MACLEAN,I.A et al. Attaching Analytes in the Proximity of the Active Site of Enzymes. J. Chem. Soc., Chem. Commun. 1992, Vol.18, pages 1283-5.	1-11 and 14 - 18

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 30 APRIL 1997	Date of mailing of the international search report 05 JUN 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PADMASHRI PONNALURI Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/02560

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,482,698 A (GRIFFITHS) 09 January 1996, column 4, line 54.	19-20